Nature and Cause of the "Cotton-Fur" Abnormality in Mink[1,2]

F. M. STOUT, J. E. OLDFIELD AND JOHN ADAIR
Department of Dairy and Animal Husbandry, Oregon State College, Corvallis

A condition known as "cotton-fur" (CF) in which the underfur of affected mink develops a drab, white color and a flimsy texture, with the longer guard hairs normally colored, was described in 1929 (Seton, '29). Occurrence of this condition causes considerable economic loss, and a mail survey conducted in 1954 indicated that it was present to some degree throughout the United States (Adair, '56). During 1940 to '43, studies at Pullman, Washington, indicated that no CF mink were obtained from matings of CF parents or interbreeding of their descendants when a "standard" mink ration was fed (Hummon and Bushnell, '43).

More recently, experiments carried out in Norway have shown that young silver foxes placed at weaning on diets high in marine fishes or fish products developed a fur characterized by a lack of pigmentation; however, fox pups fed raw meat exclusively showed normal fur without signs of greying (Ender and Helgebostad, '47). From these observations the authors postulated a deficiency associated with the fish diets. Supplementation with rice starch and glucose, Fe, Cu, Co, Zn and Mn; vitamins A, D, E, K, thiamine, pantothenic acid and C proved ineffective in preventing achromotrichia. The possibility of tyrosine deficiency was eliminated as fish in comparison with meat have been shown to be a relatively good source of this amino acid. Similarly, a lack of lysine as a causative factor was considered, but subsequently was assumed unimportant, as cod roe (which prevented symptoms of achromotrichia in foxes) is relatively low in this amino acid.

Further experiments (Helgebostad and Ender, '51) showed that considerable protection against achromotrichia could be effected by feeding a supplementary mixture of all known synthetic B vitamins or substances rich in these factors, such as cod roe, animal liver and brewers' yeast. No curative effect was noted if greying of the fur was well advanced prior to supplementation.

Later the experiments were extended to include mink (Helgebostad and Ender, '55) and the symptoms characteristic of "cotton-fur" occurred when these animals were fed diets primarily composed of "coal fish" (*Gadus virens*). The CF condition appeared to be intensified by the presence of large amounts of marine fats in the feed, which led these writers to conclude that fat peroxides acted *in vivo* to destroy certain B vitamins necessary for normal fur pigmentation.

In the United States, CF mink have often been associated commercially with the feeding of whiting, *Merluccius bilinearis*.[3] In Oregon an unexpectedly large proportion of CF mink resulted when attempts were made to use Pacific hake, *Merluccius productus*, (a marine species seldom used commercially) as a mink feed source (Adair, '55).

Data presented in this paper indicate that "cotton" mink are the product of a genetic-nutritional interaction in which susceptible mink families succumb to an unidentified heat-labile factor present in Pacific hake and whiting. Also presented are observations on the nature of the fur abnormality, means of prevention and ef-

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[2] This study was supported by a grant from the Mink Farmers' Research Foundation, Milwaukee, Wisconsin.
[3] Stephenson, R. G., 1954, personal communication.

fects of inclusion of various qualities and quantities of fats on the production of CF mink.

EXPERIMENTAL AND RESULTS

Feeding experiments with more than 1000 mink have been conducted over a 5-year period, using a basal diet of the following percentage composition: horsemeat, 7; beef liver, 4; tripe, 14; mixed sole, 42; mixed rockfish, 25; wheat germ, 2; commercial mink cereal,[4] 2; brewers' yeast, 1.5; alfalfa meal, 1.5; and steamed bone meal, 1; (whole fishes were used and ground for mixing in the diet). Experimental diets were made by replacing varying amounts of the basal fishes (generally sole) with similar weights of hake or whiting. In each year the experiments began in early July, shortly after the kits were weaned, and terminated at the time of pelting in early December. The animals were individually housed in "pelter" cages of woven galvanized wire (12 × 14 × 36 inches in size). The feed was kept refrigerated at −15°C until time of mixing and was fed once daily on the top wire of the cage.

The inclusion of either hake or whiting in the ration has resulted in a consistent production of CF mink. This production appears to be directly dependent on the percentage of these fishes comprising the diet, as is evident from figure 1, where

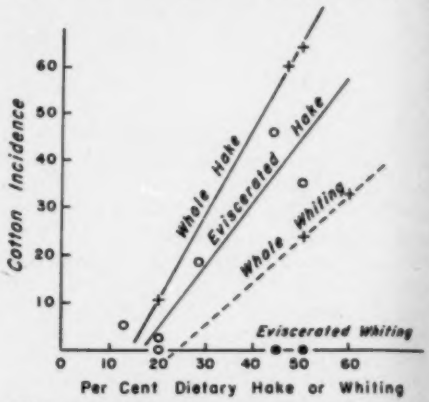

Fig. 1 Relationships of CF incidence to amounts of whole or eviscerated hake and whiting in the diet.

[4] Cer-L-Meal, produced by Crown Mills, Portland, Oregon.

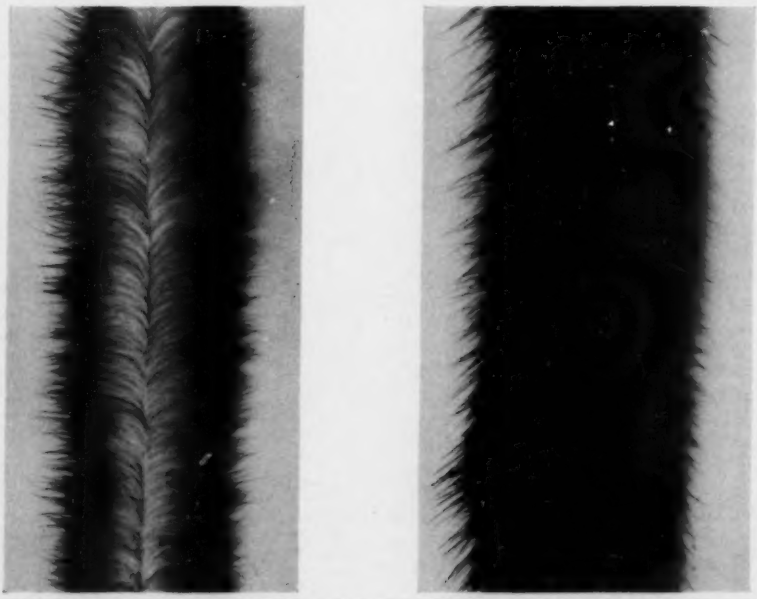

Fig. 2 Pelts of "cotton" (left) and "normal" mink, parted to show underfur.

each point on the graph represents a group of 20 to 48 mink. When the fish were cleaned and the viscera discarded the incidence of CF mink was lowered.

The external appearance of CF mink varies with the individual animals, but quite often mink so affected are small and emaciated, possessing a roughened fur coat and the characterizing "light-colored" underfur; some CF mink, however, appear to be in a healthy condition. Figure 2 presents a "cotton-fur" and a normally pigmented fur for comparison. The lack of pigment in the underfur is the most striking symptom of a large syndrome characterizing CF mink. This amount of underfur depigmentation is not typical of all CF mink, as an array of animals from those possessing an underfur almost devoid of pigment to those approaching a normal fur color have been observed (fig. 3). In many cases the depigmented underfur covers only the sides leaving a normally colored back.

Fig. 3 Fur samples illustrating variation in degree of achromotrichia encountered in mink fed a diet containing 50% of hake.

Growth curves (fig. 4) attest to the fact that CF mink are smaller than their normal counterparts, indicating induction of further anomalous metabolic conditions that affect growth. Mature CF males average 60% of the control males' weight; CF females average 86% of the weight of normal females. Growth curves for mink on a CF-genic diet which did not exhibit the symptoms of CF are intermediate between affected and control animals.

Two experiments, one in 1956 and one in 1957, were devoted to assessing the effects of rancid dietary fat on the incidence of CF mink. In 1956, 16 mink were fed the basal diet modified by adding 5% of rancid sardine oil in place of a like weight of sole, whereas 16 additional mink received the basal diet with rancid horsemeat substituted for fresh horsemeat. No CF mink occurred with either treatment, although the furs produced had a brownish cast. In 1957, 30 mink were fed the basal diet with 5% of herring oil replacing an equivalent weight of sole. Since ionizing radiation has been mentioned (Dugan, '57) as causing a high rate of fat peroxide formation, a sample of herring oil was subjected to radiation treatment at Arco, Idaho, and an additional 30 mink were fed the basal diet similarly supplemented with this irradiated oil. Again, no CF mink resulted in either case, but the fur showed a brownish coloration. The results of these 4 trials indicated that the CF condition could not be attributed to the inclusion of rancid marine or animal fats, per se, at a 5% level in the diet.

Twenty mink fed rations including 50% of hake treated by heating to 93°C in open steam cookers did not develop the CF condition, indicating that the hake-containing ration is adequate for development of normal pigmentation and that

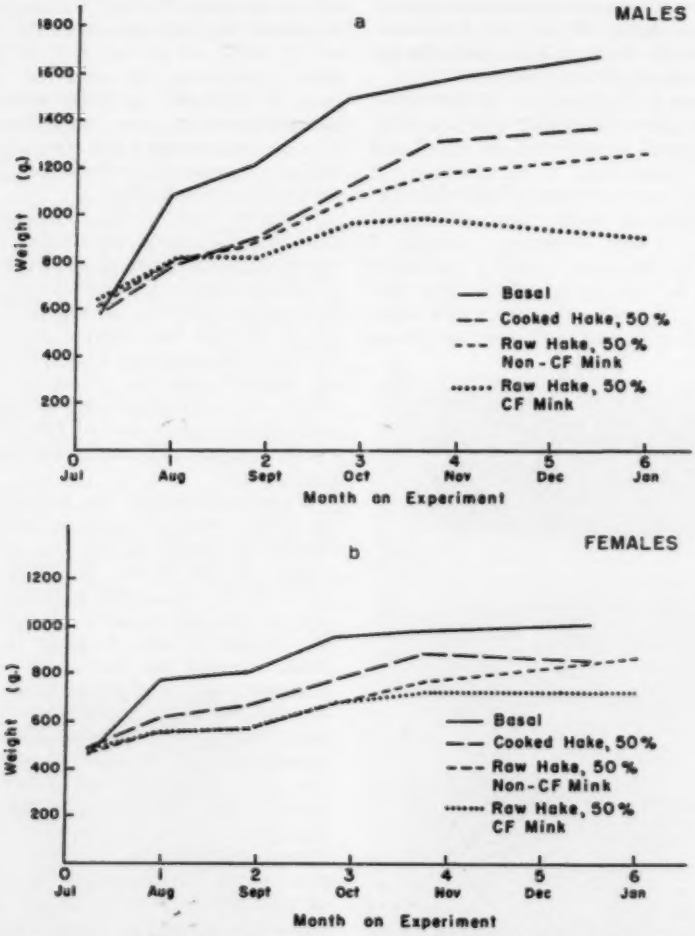

Fig. 4 Growth curves of (a) male mink and (b) female mink fed basal (77), raw hake (61) and cooked hake (20) diets, with a comparison of CF and non-CF mink fed the CF-genic (raw hake) diet. (Numbers of mink per diet shown within parentheses.)

hake contains a heat-labile, CF-causing factor. Likewise, cooked hake promotes better growth than uncooked hake; however, growth still did not approach the superior growth of the control animals (fig. 4).

The observation that all mink fed a CF-genic diet did not develop the CF condition and also that certain families were more involved suggested that susceptibility may be under genetic control. To test this hypothesis, entire litters from mink that had previously produced "cottons" and those from mink which had produced no "cottons" on a CF-genic diet were raised on a CF-genic diet. The results (fig. 5) indicate that susceptibility is largely limited to family groups and probably is genetically influenced. Attempts are in progress to establish CF-susceptible and CF-resistant strains of mink.

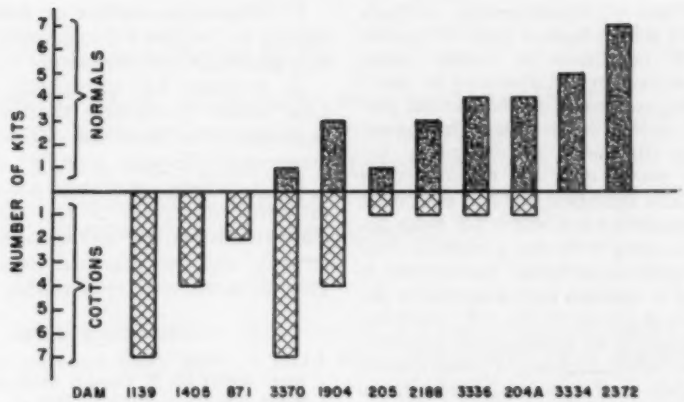

Fig. 5 Incidence of CF mink by family group. Each bar represents a litter; the stippled and cross-hatched portions indicate numbers of young within these litters which appeared "normal" or "cotton-furred," respectively.

When CF mink were transferred to the basal ration for a 6-month period (January to July), the new fur emerged normally pigmented following early summer shedding. The normal fur color was maintained in these adult animals even when they were replaced on the CF-genic ration from July through the next winter's furring period. This observation demonstrates that only young mink develop the CF condition and suggests that depigmentation of the fur occurs only when the demands for fur growth coincide with the stress of body growth.

DISCUSSION

A "cotton-fur" (CF) condition occurs frequently and consistently when hake and whiting are substituted for other fish (sole, rockfish) in an adequate basal diet for mink, and the frequency of occurrence is correlated positively with the amount of these fish fed. The different incidence of CF animals resulting from feeding similar amounts of hake and whiting suggests that these fish contain different concentrations of the CF-causative factor and this reasoning is strengthened by the observation that evisceration of whiting resulted in a complete elimination of CF, whereas evisceration of hake only reduced the occurrence. Also it is apparent that the entire fish is richer in the CF-causative factor than its eviscerated counterparts.

Since cooking the hake prior to feeding resulted in complete elimination of CF development, it is unlikely that the CF-genic ration is naturally deficient in some nutrient as postulated by Ender and Helgebostad ('47). A more likely explanation would be a dietary deficiency induced by the presence of an antagonistic heat-labile factor, possibly an enzymatic antimetabolite.

It has been established that peroxides formed from animal or marine fats during natural rancidification or those induced by ionizing radiation are not the primary cause, per se, of the CF condition. The suggestion of the Norwegian workers (Helgebostad and Ender, '55) that rancid fats may intensify the condition has not been tested, but the work reported herein suggests that the rancid fats would have to be superimposed on a CF-genic diet for this to occur.

The observation that CF animals are significantly smaller than normal-appearing animals fed the same diet is interesting. One may predict from this relationship that the CF condition is but one manifestation of a broader metabolic interference associated with the feeding of hake and whiting.

The selection of experimental animals from parents which had or had not exhibited the CF condition in earlier years revealed that certain families may be genetically predisposed to CF development, provided a CF-genic diet is fed. This latter provision is the point of difference between this work and an earlier study (Hummon and Bushnell, '43) in which no hereditary tendencies towards CF were detected when mink were fed a normal diet. Thus a genetic-nutritional interaction is evident, and it appears that progress in the practical elimination of the CF condition might be effected by appropriate selection of feeds, including cooking of those known to be CF-genic, and by selection of animal strains having no history of the fur abnormality.

Further studies are in progress designed to test the effectiveness of various purified supplements fed with a known CF-causative diet, and to concentrate and isolate the CF factor(s).

SUMMARY

1. The "cotton-fur" (CF) abnormality in mink has been produced experimentally and described.

2. Inclusion of raw, whole hake or whiting in otherwise adequate diets for mink results in appearance of CF in the animals fed. Incidence of CF can be related to the proportions of these fish in the diet.

3. Evisceration of the causative fish prior to feeding eliminated CF incidence in the case of whiting and lowered it in the case of hake.

4. Thorough cooking of hake prior to mixing in the diet led to complete elimination of the CF development.

5. Feeding 5% of animal or marine fats, under conditions conducive to fat perioxide formation did not result in occurrence of CF pelts.

6. A genetic tendency toward higher or lower incidence of CF when known causative diets are fed has been demonstrated, and the practical aspects of this genetic-nutritional relationship discussed.

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Connective Tissue Studies[1,2]

III. ASCORBIC ACID, COLLAGEN AND HEXOSAMINE DISTRIBUTION AND HISTOLOGY OF CONNECTIVE TISSUE IN SCARS PRODUCED IN GUINEA PIGS ON VARIOUS VITAMIN C DIETARY LEVELS FOLLOWING WOUNDING BY ABDOMINAL INCISION

ARTHUR F. ABT, SUSANNE VON SCHUCHING AND JOSEPH H. ROE
Radioisotope Service, Veterans Administration Center, Martinsburg, West Virginia

Aschoff and Koch, in 1919, were the first to note that the basic factor in human scurvy was an alteration of the intercellular connective tissue. In the following year, Hess ('20) described the tissue changes in scurvy in his classical monograph, Scurvy, Past and Present.

In 1924, Höjer in a most comprehensive report, augmented the description of the tissue changes in scurvy noted by Hess. Probably the most important finding reported by Höjer was that in scurvy the rate of collagen formation is diminished and that a widespread deficiency of collagen exists in the connective tissue. Bourne ('42) has also noted a diminished rate of formation of collagen in scurvy. In 1926, Wolbach and Howe first observed that newly formed fibrous tissue in wound repair is free from collagen, whereas they found no striking abnormality of the collagen fibers of connective tissue already existing in scorbutic guinea pigs.

In previous studies we have reported a direct relationship between the amount of vitamin C in the diet and the strength of the healing wound, as well as between the dietary level of ascorbic acid and its concentration in scar tissues, in the tissues of other organs and in the blood.

For the past several years we have likewise studied the role of vitamin C in the formation and maintenance of connective tissue. These studies have been made in the guinea pig both on *recently* healed abdominal wounds, reported by Abt, et al. ('59a), as well as on similar *long healed* wounds, reported by Abt and coworkers ('59b).

We have previously demonstrated that ascorbic acid is essential for (a) *the production* of connective tissue to ensure immediate postoperative healing, as well as for (b) *the maintenance* of connective tissue in previously formed scar tissue. We have also shown that ascorbic acid accumulates in scar tissue immediately following wounding and persists for long periods of time.

A part of our study has been directed to measuring in scar tissue the principal constituents of connective tissue: collagen, and ground substance. We have used determinations of hydroxyproline as a measure of collagen and determinations of hexosamine as a measure of ground substance at varying levels of dietary ascorbic acid intake.

MATERIALS AND METHODS

Young male guinea pigs of the Hartley strain, weighing 220 to 250 gm, were used. Similar dietary management was instituted and the abdominal operative technique was performed as previously reported by Abt, von Schuching and Roe ('59a). Tissues

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[2] This investigation was supported in part by Research Grant USPHS A-1949(C1) from the NIH, Public Health Service.

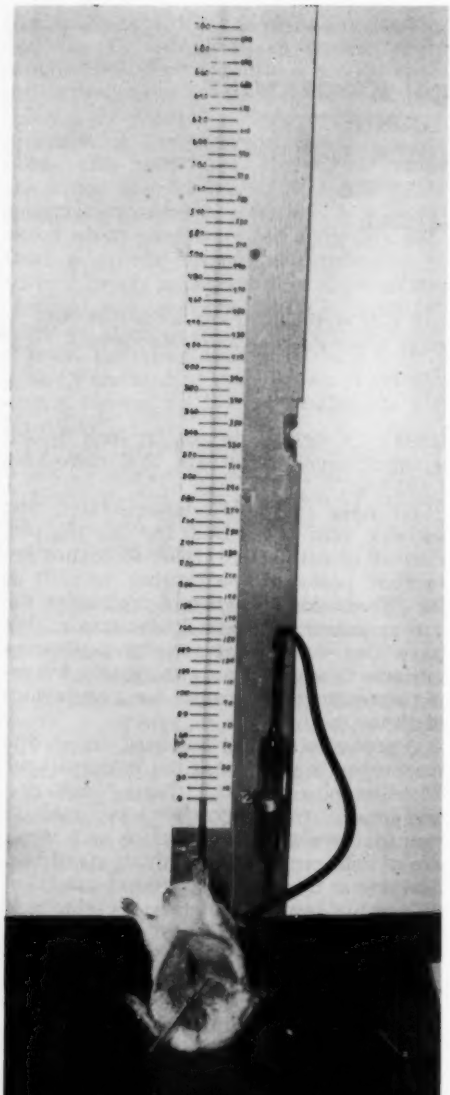

Fig. 1 Illustrates the Mercury Manometer used to test the pressure of rupture of the wound in the anesthetized guinea pig.

for analysis were taken immediately after the wound rupture determinations. Figure 1 illustrates the mercury manometer used to test the pressure of rupture of the wound in the anesthetized guinea pig. Mucopolysaccharides were determined as hexosamine by the method of Elson and Morgan ('33), as modified by Roseman and Daffner ('56). Collagen was estimated as hydroxyproline by the modification by Martin and Axelrod ('53) of the method of Neuman and Logan ('50). The ascorbic acid content of blood and tissues was determined by the method of Roe and Keuther ('43). Histologic sections were fixed in formalin or Bouin's fluid and stained for connective tissue by Foot's modification of Masson's Trichrome stain.

RESULTS

Ascorbic acid distribution. We have previously noted that the concentration of ascorbic acid in the wound tissue rose above the concentration in distant tissue in recently healed wounds. We have further noted that this higher concentration of ascorbic acid persists in scar tissue after long intervals of healing. Following these original observations we have further fractionated the scar tissue of abdominal wounds for ascorbic acid concentration, to determine in exactly which tissue the highest level of ascorbic acid existed.

We have separated the central core of the scar which consists nearly exclusively of connective tissue and termed this the "scar core connective tissue." The tissue adjacent to the wound and not more than 1 to 2 mm from it, we have designated the "paravulneral tissue" (Stein and Wolman, '58). From the same animal, samples of abdominal muscle were also taken 0.7, 1.5 and 3 cm from the edge of the wound, as well as of muscle from the leg and back. Connective tissue was obtained from the achilles and patellar tendon and from the ligamentum nuchae.

The highest values for ascorbic acid from these enumerated tissues, were obtained from the scar core connective tissue and from the connective tissue of the tendons and ligaments. Thus, the increase of ascorbic acid in scar tissue after wounding is definitely linked to the connective tissue. Our results are noted in figure 2.

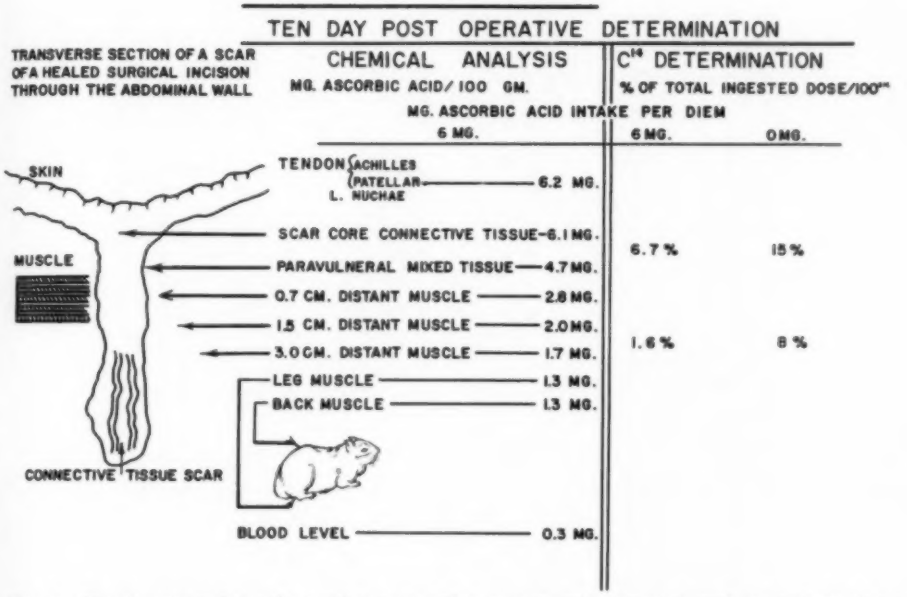
ANIMAL EXPERIMENTS
TEN DAY POST OPERATIVE DETERMINATION
TRANSVERSE SECTION OF A SCAR OF A HEALED SURGICAL INCISION THROUGH THE ABDOMINAL WALL
CHEMICAL ANALYSIS
MG. ASCORBIC ACID/ 100 GM.
C14 DETERMINATION
% OF TOTAL INGESTED DOSE/100gm
MG. ASCORBIC ACID INTAKE PER DIEM
6 MG.
6 MG.
0 MG.
SKIN
MUSCLE
CONNECTIVE TISSUE SCAR
TENDON (ACHILLES (PATELLAR L. NUCHAE 6.2 MG.
SCAR CORE CONNECTIVE TISSUE 6.1 MG.
PARAVULNERAL MIXED TISSUE 4.7 MG.
0.7 CM. DISTANT MUSCLE 2.8 MG.
1.5 CM. DISTANT MUSCLE 2.0 MG.
3.0 CM. DISTANT MUSCLE 1.7 MG.
LEG MUSCLE 1.3 MG.
BACK MUSCLE 1.3 MG.
BLOOD LEVEL 0.3 MG.
6.7 %
15 %
1.6 %
8 %

Fig. 2 Fractionation of ascorbic acid in scar and distant muscle, chemical analysis, and C^{14} determination. The most important finding is the highest concentration of ascorbic acid in the scar connective tissue. Marked differences are also noted in the retention of C^{14} in the tissues of normal and scorbutic animals.

Collagen and hexosamine distribution. In table 1 the collagen content, calculated as hydroxyproline, in gm/100 gm of dry tissue is shown. The following tissues were analyzed: abdominal skin, abdominal muscle, scar skin, scar muscle, teeth, leg bone and cartilage. Patellar cartilage was analyzed in the experiment using a commercial chow[3] as the dietary source, whereas in the animals receiving the semi-synthetic diet of Reid and Briggs ('53), costal cartilage was analyzed. Statistical analysis showed no significant differences in collagen content between the control and scorbutic groups except in the costal cartilage of those fed the scorbutic diet for 19 days. In view of the failure to observe significant differences in all of the other analyses, the latter finding is probably the result of technical error.

Table 2 shows the hexosamine content in milligrams per 100 gm of dry tissue, or milligrams per 100 ml for serum. Values for abdominal skin, abdominal muscle, scar skin, scar muscle, leg bone, cartilage and serum were determined. No significant differences in values between control and scorbutic groups were found.

When a comparison is made between the hexosamine values of the normal tissue at the time of operation, and the values for scar tissue in the operated area at increasing intervals after the operation, a rise is noted in the amount of hexosamine in the scar tissue in both control and scorbutic animals in all instances except for the data for three days after operation. The increases were statistically significant in most cases.

Summarizing the results obtained (tables 1 and 2), neither collagen nor hexosamine levels showed a significant difference between the control and scorbutic groups. The collagen values were compared at intervals after the operation. Except for a decrease in the skin value three days after operation, no other changes were observed. At 6 days after operation, the

[3] Purina Chow.

TABLE 1

Collagen content, converted from hydroxyproline analyses, of wound and other tissues in guinea pigs fed commercial chow and semi-synthetic diets, with and without ascorbic acid. Data represent averages of 5 animals per series. Animals were on identical diets, control guinea pigs receiving 90 mg of ascorbic acid per animal daily. Values are gm per 100 gm of dry tissue

Daily oral intake of vitamin C	Total days on diet	At operation		At sacrifice					
		Abdominal skin	Abdominal muscle	Days post-operative	Scar skin	Scar muscle	Teeth	Leg bone	Cartilage
mg	*no.*			*no.*					
				Commercial chow diet[1]					
100 by dropper	20	—	—	10	36.7 ± 4.98[2]	11.3 ± 1.36	10.18 ± 2.80	9.66 ± 0.38	25.6 ± 5.18[3]
0	20	—	—	10	30.0 ± 2.35	11.0 ± 1.95	9.80 ± 4.05	9.18 ± 1.63	31.2 ± 3.05
P of difference		—	—		0.02	> 0.5	> 0.5	> 0.5	0.1
				Reid-Briggs semi-synthetic diet					
90 mixed in diet	13	51.8 ± 2.67	10.2 ± 3.62	3	38.4 ± 4.58	7.9 ± 2.79	13.50 ± 1.30	13.30 ± 1.20	22.5 ± 2.08[4]
0	13	48.3 ± 4.92	8.7 ± 1.10	3	36.7 ± 0.70	8.5 ± 1.58	13.80 ± 0.70	12.10 ± 1.66	24.0 ± 1.36
P of difference		0.2	0.4		> 0.5	> 0.5	> 0.5	0.2	0.3
90 mixed in diet	16	38.3 ± 7.75	10.5 ± 1.68	6	38.4 ± 5.04	8.0 ± 0.81	—	—	—
0	16	43.1 ± 3.10	11.2 ± 1.94	6	38.1 ± 4.18	8.3 ± 0.89	—	—	—
P of difference		0.24	> 0.5		> 0.5	> 0.5	—	—	—
90 mixed in diet	19	40.2 ± 8.02	9.0 ± 2.01	9	32.2 ± 4.14	13.0 ± 2.38	11.40 ± 0.70	11.70 ± 1.28	22.0 ± 1.22
0	19	47.0 ± 8.86	6.1 ± 2.28	9	30.9 ± 2.98	9.6 ± 1.81	12.40 ± 0.85	11.80 ± 0.70	25.5 ± 1.37
P of difference		0.24	0.08		> 0.5	0.04	0.08	> 0.5	< 0.010
90 mixed in diet	22	41.9 ± 4.51	11.6 ± 2.16	12	35.5 ± 5.52	10.5 ± 0.26	12.60 ± 0.86	12.20 ± 1.38	22.9 ± 3.31
0	22	40.5 ± 3.00	11.2 ± 2.55	12	34.2 ± 5.51	11.1 ± 4.20	11.60 ± 0.47	12.30 ± 0.59	24.0 ± 2.73
P of difference		> 0.5	> 0.5		> 0.5	> 0.5	0.05	> 0.5	> 0.5

[1] Purina chow. [2] Standard deviation. [3] Patellar. [4] Costal.

TABLE 2

Hexosamine content of wound and other tissues in guinea pigs fed commercial chow and semi-synthetic diets, with and without ascorbic acid. Data represent averages of 5 animals per series. Animals were fed identical diets, control guinea pigs receiving 90 mg of ascorbic acid per animal daily. Values are mg per 100 gm of dry tissue or mg per 100 ml of serum

Daily oral intake of vitamin C	Total days on diet	At operation		At sacrifice					
		Abdominal skin	Abdominal muscle	Days post-operative	Scar skin	Scar muscle	Leg bone	Cartilage	Serum
mg	*no.*			*no.*					*mg/100 ml*
					Commercial chow diet[1]				
100 By dropper	20	—	—	10	352.0 ± 86.0[2]	—	345.0 ± 310.0	511.0 ± 249.0[3]	75 ± 47.0
0	20	—	—	10	327.0 ± 66.0	—	219.0 ± 166.0	608.0 ± 107.0	116 ± 75.0
P of difference					> 0.5		0.3	> 0.5	0.2
					Reid-Briggs semi-synthetic diet				
90 Mixed in diet	13	372.4 ± 75.0	247.6 ± 76.0	3	357.8 ± 78.0	442.8 ± 129.0	287.8 ± 20.8	270.4 ± 60.0[4]	129 ± 33.0
0	13	362.0 ± 42.5	181.3 ± 129.0	3	378.8 ± 49.4	430.2 ± 140.0	263.6 ± 23.2	245.0 ± 24.0	103 ± 43.4
P of difference		> 0.5	> 0.5		0.5	> 0.5	0.5	> 0.5	0.5
90 Mixed in diet	16	235.4 ± 42.5	207.8 ± 54.6	6	448.0 ± 38.4	378.0 ± 114.0	—	—	85 ± 21.5
0	16	319.8 ± 84.0	190.8 ± 138.0	6	479.8 ± 79.0	329.8 ± 128.0	—	—	90 ± 17.6
P of difference		> 0.5	> 0.5		> 0.5	> 0.5			0.5
90 Mixed in diet	19	291.8 ± 59.0	219.8 ± 117.0	9	471.6 ± 50.0	456.8 ± 50.8	321.2 ± 69.0	264.9 ± 44.0	66 ± 26.7
0	19	362.0 ± 60.0	234.8 ± 195.0	9	434.4 ± 58.0	469.2 ± 56.7	267.2 ± 65.5	214.6 ± 35.5	120 ± 39.8
P of difference		> 0.5	> 0.5		> 0.5	> 0.5	> 0.5	> 0.5	> 0.5
90 Mixed in diet	22	280.2 ± 52.6	173.8 ± 93.0	12	431.6 ± 15.3	377.8 ± 222.1	351.8 ± 56.1	208.3 ± 12.2	69 ± 29.5
0	22	264.0 ± 120.0	287.0 ± 120.0	12	598.2 ± 240.0	434.8 ± 65.9	280.2 ± 48.2	231.4 ± 34.8	71 ± 37.2
P of difference		> 0.5	> 0.5		> 0.5	> 0.5	> 0.5	> 0.5	> 0.5

[1] Purina chow. [2] Standard deviation. [3] Patellar. [4] Costal.

TABLE 3

Analyses of collagen, determined from hydroxyproline, and of hexosamine in carrageenin granulomata 10 days after subcutaneous injection of carrageenin in guinea pigs. Data represent averages of 5 animals per series

Average values	Daily intake of ascorbic acid	
	90 mg	0 mg
Collagen, gm/100 gm dry tissue	3.8 ± 1.3	4.0 ± 1.4
Hexosamine, mg/100 gm dry tissue	1216 ± 63.6	949 ± 4.1

absolute amounts of collagen in skin were exactly the same as values at operation.

The hexosamine values showed absolute increases after the third postoperative day in both the control and scorbutic groups. The hexosamine values were significantly higher in the control group at the 6th, 9th, and 12th postoperative days than at the day of operation. The scorbutic animals showed increased hexosamine values at the same periods, though with a wider range.

In table 3 are noted the average of analyses of collagen, calculated from hydroxyproline, and of hexosamine determined on specimens from carrageenin granulomata.[4] They were taken 10 days after the subcutaneous injection of carrageenin into guinea pigs receiving daily intakes of 90 mg of ascorbic acid and no ascorbic acid. No difference was noted in the collagen content of the carrageenin tumor for the animals on the 90-mg daily intake of ascorbic acid and those on the scorbutic diet.

The average hexosamine values of specimens from the carrageenin tumor were greater for the animals on 90-mg daily ascorbic acid intake, than for the average of the group deprived of ascorbic acid.

[4] We are indebted to Mr. Leonard Stoloff, Research Director, Seaplant Corporation, New Bedford, Massachusetts, for generously supplying us with a purified extract of Irish moss (*Chondrus crispus*).

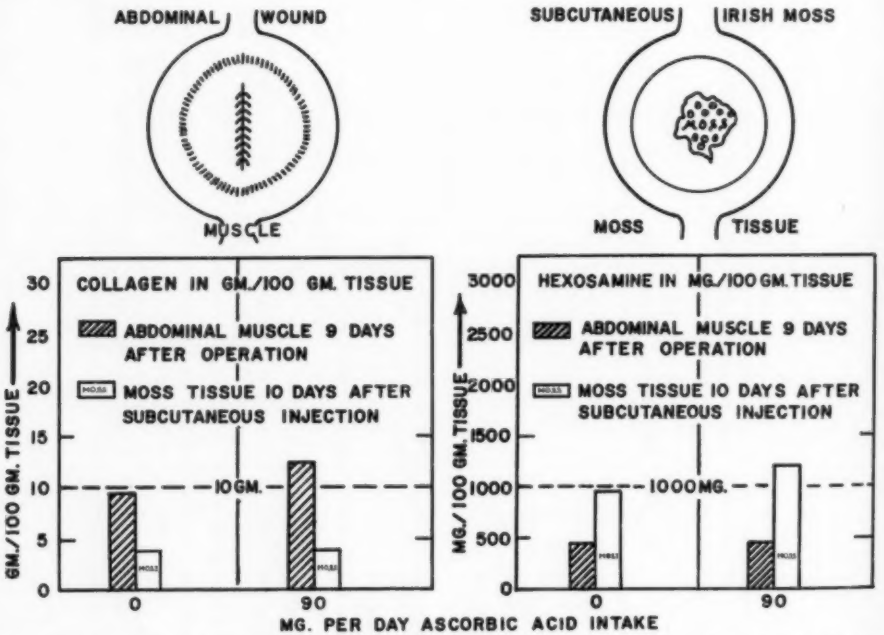

Fig. 3 Comparison of collagen and hexosamine levels in scar wound muscle and subcutaneous carrageenin granuloma at 0 and 90 mg of daily ascorbic acid intake. Determinations were made 9 days after operation and 10 days after subcutaneous injection of the carrageenin. Data represent averages for 5 animals.

Fig. 4 Cross section of an abdominal incision in the guinea pig 10 days postoperative. Animal was on a maintenance diet of 6 mg ascorbic acid daily. Foot's modification of Masson's Trichrome Stain. × 10.

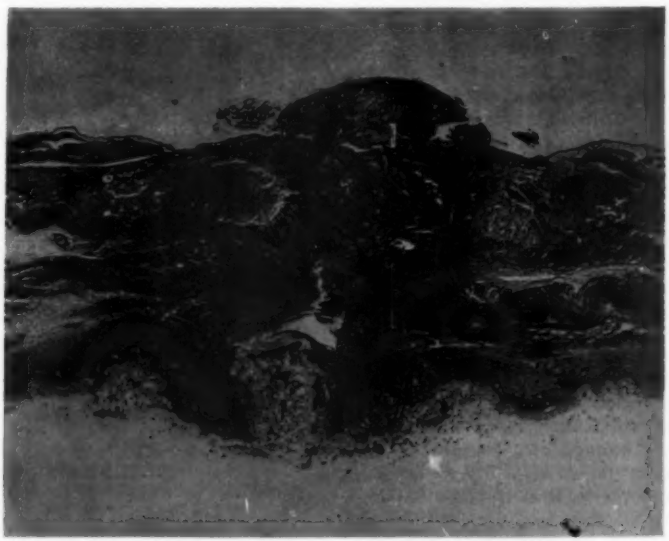

Fig. 5 Cross section of an abdominal incision 18 days postoperatively in a guinea pig on a scorbutic diet. Note failure of epithelium to heal and lack of formation of new connective tissue. Foot's modification of Masson's Trichrome Stain. × 10.

In figure 3 is shown a comparison of collagen and hexosamine levels in scar wound muscle and subcutaneous carrageenin granuloma at zero and 90-mg daily ascorbic acid intake. Determinations were made 9 days after operation and 10 days after subcutaneous injection of the carrageenin. The values for collagen are lower in the granuloma tissue than in the scar muscle, both in the scurvy group and in the group receiving optimal vitamin C intake, at equivalent time periods following operation and injection of carrageenin.[5] The levels of hexosamine are the reverse of the collagen levels when comparison is made at equivalent times for the two diets, both hexosamine levels being higher in the granuloma.

Histology of connective tissue. Cross section specimens of the scars were made as they were removed from the animals receiving the varying dietary vitamin C intakes. These were stained with ordinary hematoxylin and eosin stains and with special connective-tissue stain used to demonstrate the regeneration of fibers at various intervals following the operation and for animals on different dietary intake levels. Figure 4 is a cross section of a healing scar taken 10 days postoperatively from a guinea pig who had been receiving 6 mg of ascorbic acid daily, orally, a maintenance diet. The epithelium has healed over completely and the line of incision has filled in with numerous new connective tissue fibers. In contrast to this picture of an active regenerating wound, figure 5 represents a cross section of a scar from a scorbutic animal, 18 days postoperatively. The epithelial layer remains separated where the incision occurred and the derma is ununited. The central portion seems still to contain fibrin and intracellular substances. There are very few, if any, new connective tissue fibers. The dearth of new collagen formation as noted by the special stain employed is most marked when contrasted to the histologic cross section of the healing wound of a guinea pig on a maintenance vitamin C intake. The histologic findings in the guinea pig here presented parallel in every detail histologic findings on wound healing in vitamin C-depleted human subjects, previously observed by one of the authors, Arthur F. Abt[6] (Wolfer et al., '47).

DISCUSSION

A most important finding here reported, is the demonstration that *the highest concentration of ascorbic acid in the wound is in the connective tissue.* This concentration is equal to its concentration in the connective tissue of the tendons and ligaments, simultaneously examined. The exact mechanism related to the increased concentration and build-up of ascorbic acid in wound connective tissue is unknown.

We conclude from our results that hydroxyproline determinations are not an accurate reflection of collagen production; further, that hexosamine is derived largely from plasma, and its concentration in scar tissue is independent of scurvy. The histologic studies corroborate the chemical findings and substantiate the conclusion that ascorbic acid is necessary for the production of new connective tissue.

According to Kellgren ('52), the connective tissues perform at least two important functions. First, they provide a tough framework for the body, giving it cohesion and form, and also a system of levers which allow the exertion of considerable mechanical force; and, secondly, they provide the lubricating materials which enable the various parts of the body to move smoothly and efficiently upon each other. It is also possible that they may play an important part in metabolism, but this possibility still remains unexplored.

According to Stetten ('51), the function of collagen is simply to exhibit a high tensile strength; no other function is known.

The role of collagen in wound healing, and particularly in relation to the healing

[5] Since the manuscript for this paper was submitted for publication, a paper by Chozo Mitoma and Thomas E. Smith has appeared, "Studies on the Role of Ascorbic Acid in Collagen Synthesis," J. Biol. Chem., 235: 426–428, February 1960. Using L-proline-C^{14} as tracer for the formation of collagen in carrageenin granuloma, Mitoma and Smith found that conversion to hydroxyproline was not affected by ascorbic acid deficiency.

[6] "Methods for Detection of Early Signs of Vitamin C Deficiency," investigation under Contract OEMcmr no. 71, Committee on Medical Research, March 25, 1944, responsible investigators, Chester J. Farmer, M.D. and Arthur F. Abt, M.D. Also separately reported.

of wounds in scurvy, has been studied for a considerable period. Wolbach ('37) noted that the chief function assigned to vitamin C in the body is concerned with the formation of colloid intercellular substances. Abt and Farmer ('38) noted that the intercellular substances which appear to be regulated by vitamin C are of mesenchymal origin—the collagen of all fibrous tissue structures, all non-epithelial cement substances, including the intercellular substance of the capillary wall, dentin, cartilage and the matrices of bone.

In relating scurvy to wound healing, Sylvia Bensley ('34) characterized all healing processes by three stages: (1) edema, (2) gelation and (3) fiber production—pointing out that in scurvy the process stopped at the first stage, namely, edema.

Klemperer ('55) notes that the mechanism of deranged collagen formation in scurvy is as yet unclarified, and Goldsmith ('53) agrees that the exact nature of the defect is controversial. Gersh ('52) has noted that in acute scurvy, morphologic collagen shows a decrease, whereas, chemically, no difference exists between that found in non-scurvy animals.

Elster ('50) states that the maintenance of collagen does not require ascorbic acid, and Robertson and Schwartz ('53) note that the formation, in contrast to the maintenance of collagen, requires ascorbic acid.

Lloyd and Sinclair ('53) point out that it is generally agreed that connective tissue is defective in scurvy, though there is disagreement on the nature of the defect, one group noting intactly functioning cells, but that ascorbic acid is ultimately necessary for the setting or fibrillation of collagen and similar substances, while the other group, represented by Fish and Harris ('34), and Ham and Elliott ('38), regards the defect as being in the function of the connective tissue cell.

In recent studies Gould and Woessner ('57) concluded that the collagen formation is impaired as early as 4 days after withdrawal of ascorbic acid. In a subsequent communication Gould ('58) notes that apparently rapid collagen biosynthesis, such as that involved in tissue repair, may be mediated directly by ascorbic acid.

Kodicek and Loewi ('55) reported that collagen formation was impaired in vitamin C deficiency as evidenced by low hydroxyproline values and they further noted, in agreement with our findings, the values for hexosamine in scorbutic granulation tissue did not differ from the values obtained in control animals.

Edwards and Dunphy ('58) note that the hexosamine may be largely derived from plasma glycoproteins.

Weiss ('59) has noted that erstwhile round connective tissue cells elongate along the lines which fibrin strands assume in fresh wounds. Thus the collagen fibers of the new connective tissue of the healing wound are laid down in parallel strands.

Many investigations have been reported concerning the precursor of collagen—termed "procollagen"—since the early experiments of Zacharidès (1900) and Nageotte ('27), who extracted collagen from rat-tail tendon with dilute acid and salt. The latter author termed the soluble fraction "précollagène" and believed it was a soluble precursor of insoluble fibers. Jackson ('58) and Green and Lowther ('59) give most excellent recent reviews of collagen precursors.

Recently, Robertson, Hiwett and Herman ('59) have reported on the relation of ascorbic acid to the conversion of proline to hydroxyproline, and Gross ('59) has noted the effect of vitamin C deficiency on the neutral salt-extractable collagen of skin. He concludes that the deficiency of ascorbic acid either interferes with the synthesis of new collagen in intact skin or causes its destruction and removal as rapidly as it is produced.

The new connective tissue produced by the subcutaneous injection of carrageenin increases rapidly to a maximum 7 to 14 days following injection, but, unlike the connective tissue formed by wounding, is later absorbed after a period of weeks, so that after the 6th week, only a small amount of fatty tissue remains at the injected site. This resorption of connective tissue corresponds to the body response to the presence of a foreign body and differs from the formation of scar tissue after wounding.

Gillman ('57) has pointed out that the fibrosis noted in carrageenin granuloma differs considerably from that usually seen

in healing wounds. In his opinion the two types of fibroses, namely, that seen in scar formation and that produced in granuloma, are quite different. This appears to be a valid criticism and differences in data, obtained from scars produced by abdominal incision and granuloma produced by subcutaneous injection of foreign bodies such as carrageenin, should be recognized. The data obtained from these two types of scar-tissue production may not be comparable.

SUMMARY

1. We have extended previous observations on the concentration of ascorbic acid in scar tissue to a further fractionation of the tissues composing the scar and have found that *the highest concentration of ascorbic acid is in the connective tissue.*

2. The high concentration of ascorbic acid in the connective tissue of the wound parallels the concentrations of ascorbic acid found simultaneously in the connective tissue of tendons and ligaments. The next highest concentration was found in the paravulneral tissue adjacent to the scar and lower amounts occurred in specimens of abdominal muscle distant from the scar as well as samples of muscle taken from the leg and back.

3. From determinations of collagen and hexosamine content of abdominal scar tissue, and of tissue produced in carrageenin granuloma, we have shown that insignificant differences occur between animals with scurvy and those placed on saturation dietary intakes of ascorbic acid. We have also noted that postoperatively, hexosamine values increased, both in the scorbutic and control animals.

4. In histologic cross sections of abdominal incisions, new connective tissue fibers have been demonstrated by special stain in guinea pigs on a maintenance dietary level of ascorbic acid. A dearth of new connective tissue fibers occurred in histologic cross sections of abdominal scars taken from scorbutic animals. These histologic findings parallel those previously made in studies on wound healing in vitamin C-depleted human subjects.

ACKNOWLEDGMENT

Appreciation is expressed to Charles Derr who carried out the greater part of the computations and analytical determinations, as well as for the technical assistance of Carl Karickhoff; also to June Engle for helping to compile the data. We also appreciate the preparation of the photomicrographs by Harry Busey.

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Effects of Amino Acids on the Excretion of Various Proteins by the Rat[1]

R. W. F. HARDY,[2] V. O. TANTENGCO[3] AND C. A. BAUMANN
Department of Biochemistry, University of Wisconsin, Madison

Whereas there is a general parallelism between nitrogen intake and the excretion of urinary protein by the adult male rat (Rumsfeld, '56 and Finlayson and Baumann, '56) dietary glycine has been observed to cause a substantial decrease in proteinuria (Hardy and Baumann, '59). In the present study the effects of each of the other common amino acids on proteinuria were determined, and quantitative measurements were made of the individual proteins after separation by paper or starch-gel electrophoresis, or ion exchange chromatography.

EXPERIMENTAL

Protein, urea and α-amino nitrogen in urine. Male adult Holtzman rats weighing approximately 400 gm were maintained on the following basal diet throughout the experiment: 20% casein, 71% dextrin, 5% corn oil, 4% Wesson salts, 0.1% choline chloride, 0.1% vitamin mixture[4] and 0.09% vitamin E solution.[4] Vitamins A and D were supplied in one drop of halibut liver oil per week per rat. The rats were placed under light ether anesthesia and were given 10 ml of amino acid solution by stomach tube; the doses of the various amino acids were isonitrogenous (0.14 gm [0.01 mole] of N in 10 ml). The difficultly-soluble amino acids were dispersed by a minimal addition of sodium hydroxide. The administration of an equivalent amount of base neutralized by acid produced no significant effect on proteinuria, nor did the amount of water administered,[5] this latter being in agreement with results of water-loading experiments by Finlayson and Baumann ('56).

Urine collections were made for 24-hour periods and prepared for analysis as described previously; protein was determined as N in the trichloroacetic acid precipitate (Hardy and Baumann, '59), urea by the method of Archibald, ('45), and α-amino nitrogen by a modification of the method of Pope and Stevens ('39).[6] The animals were fasted during collection. Collections from all animals were made approximately three days prior to treatment, during the 24-hour period after the administration of the amino acid, and also three days after treatment. Rats that showed abnormal protein excretion during the pretreatment period were discarded. The post-treatment collection indicated the persistence of any decrease or increase observed during treatment.

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[4] The vitamin mixture consisted of the following in milligrams: inositol, 100; calcium pantothenate, 20; niacin, 10; menadione, 10; riboflavin, 2; thiamine, 2; pyridoxine, 2.5; biotin, 0.1; folic acid, 0.2; vitamin B_{12}, 0.02; and glucose to 1.0 gm; the vitamin E solution contained 50 mg of α-tocopherol/ml of corn oil.

[5] Hardy, R. W. F. 1960 Ph.D. Thesis, University of Wisconsin, Madison.

[6] Colorimetric modification of the copper method of Pope and Stevens ('39); unpublished data of C. C. Clayton, B. F. Steele and H. W. Rumsfeld. The solubilized copper is treated with tetraethylenepentamine to form a deep-blue color, the density of which is determined at 660 mμ. Since the color intensity/mole of amino acid varies somewhat between the different amino acids, an average constant has been used for conversion of optical densities obtained with an Evelyn colorimeter to α-amino N values, i.e., $K = L/C$ when $K = 2.64$, $L = 2 - \log G$, and $C =$ μg of α-amino N in 2.5 ml of reaction mixture.

Paper electrophoretic separation. Twenty-four-hour urine collections from rats were filtered through Whatman no. 42 filter paper, dialyzed exhaustively in sausage casings[7] against distilled water at 3°C, pervaporated at room temperature until a protein concentration of approximately 1% was obtained, and equilibrated with 0.01 M, pH 8.6 Tris acetate buffer. For paper electrophoresis 10 to 20 µl were applied as a narrow band to horizontal paper strips of Whatman 3 MM paper, 1 cm wide and 30 cm long. The paper strips had previously been dipped in buffer, blotted and allowed to equilibrate in the apparatus. The electrophoretic chamber consisted of a multi-compartmented plastic box 2 × 10.5 × 4 inches constructed from a container for fishing tackle. The platinum electrode compartments were joined to the buffer compartments by filter paper bridges. Six strips were run simultaneously for 4 hours at a constant voltage of 350 volts and 3 to 5 milliamperes. The dried strips were stained according to the method of Jencks et al. ('55). Mobilities of the stained urinary proteins were compared with those of plasma obtained from animals treated similarly. The strips were then cut into sections containing proteins with the mobilities of albumin, α-globulins, β-globulins and γ-globulins. The bound dye was eluted with 0.01 N aqueous sodium hydroxide and the transmission measured at 590 mµ (Block et al., '58). The relative percentages of the proteins were expressed as the relative percentages of the bound dye. Other strips were stained with Sudan black (Swahn, '52) to detect lipid or with basic fuchsin (Köiw and Gronwall, '52) to detect carbohydrate.

Starch-gel electrophoretic separation. Urinary protein for starch-gel electrophoresis was prepared as indicated for paper except that 0.05 ionic-strength Tris acetate was used. The starch[8]-gel block 30 × 20 × 1.5 cm was prepared according to the method of Sehon et al. ('56); 5 ml of approximately 1% urinary proteins slurried with starch were then placed into a ditch 0.6 cm wide, 1.5 cm deep and 18 cm long located 8 cm from the cathode and 22 cm from the anode. After one hour of equilibration in a cold room at 3°C, 300 to 400 volts were applied across the 30 cm for 18 to 24 hours. The resultant current was approximately 50 to 80 ma. The starch block was then cut parallel to the ditch into 1 cm sections and a 4 cm portion removed from the center of each section. The protein was quantitatively eluted from each of these small portions by the method of Sehon et al. ('56). The eluted protein was determined colorimetrically with Folin-Ciocalteu reagent ('27) and the optical densities plotted against section number.

RESULTS

Urinary α-amino nitrogen and urea. Male rats given 140 mg doses of the different amino acids excreted markedly different amounts of α-amino acids in the urine (table 1). Amounts above 10 mg of α-amino N/rat/day followed the administration of DL-serine, D-serine, DL-methionine, DL-isoleucine, DL-threonine, DL-tryptophan, DL-leucine, DL-aspartic, DL-alanine, DL-valine, L-proline and sarcosine. Only slight increases in α-amino N, less than 3 mg/rat/day, followed the administration of L-cysteine, L-cystine, DL-phenylalanine, L-tyrosine, L-arginine, L-serine or glycine, whereas intermediate increases followed the administration of the other amino acids. Absolute significance cannot be ascribed to the values obtained, since certain amino acids yield more color per mole of amino acid than others. In general, however, the losses of amino acids into the urine accounted for only a small fraction of the dose administered, the highest losses resulting after the administration of DL-amino acids, namely, losses of 16 and 10 mg after DL- or D-serine, respectively, and only 2.6 after L-serine. Metabolism of the N in the administered amino acids is shown by the substantial increase in urea excretion which generally occurred (table 1); DL- or D-serine were notable exceptions.

Amounts of urinary protein. Protein excretion was greatest for male rats which had received DL-serine, D-serine and L-cysteine·HCl, exceeding 9.00 mg of N/rat/day, whereas the control groups excreted an average of only 2.84 mg of N/rat/day (table 1). An elevation in protein excretion after the administration of

[7] Visking Corporation, Chicago.

[8] Starch for chromatography obtained from Amend Drug and Chemical Company, New York.

TABLE 1

Effect of various amino acids on the urinary excretion of α-amino nitrogen, urea and protein

Amino acid		Sex	α-Amino N		Urea		Protein		Protein N × 10²/urea N	
			Test	Control	Test	Control	Test	Control	Test	Control
	gm/rat		*mg/rat/day*		*mg/rat/day*		*mg/rat/day*			
DL-Serine	1.05	M	20.1	4.02	145	352	11.45	2.80	15.45	1.71
DL-Serine	1.05	F	34.2	0.89	283	265	11.75	1.27	8.88	1.03
D-Serine	0.52	M	14.3	4.02	148	352	12.35	2.80	17.76	1.71
L-Cysteine·HCl	1.57	M	2.53	1.25	406	379	9.10	3.11	4.79	1.67
L-Histidine·HCl	0.64	M	8.81	2.26	511	347	5.21	3.08	2.18	1.91
L-Lysine·HCl	1.84	M	8.26	2.26	431	347	4.04	3.08	2.01	1.91
L-Cystine	1.20	M	3.39	1.25	435	379	3.82	3.11	1.88	1.67
DL-Phenylalanine	1.65	M	2.22	1.18	399	324	3.47	2.89	1.86	1.91
DL-Methionine	1.49	M	37.4	1.25	407	379	3.42	3.11	1.80	1.67
L-Tyrosine	1.81	M	2.03	1.18	499	324	4.12	2.89	1.78	1.91
DL-Isoleucine	1.31	M	48.7	1.35	399	293	3.09	2.07	1.65	1.52
DL-Threonine	1.19	M	63.7	1.18	477	324	3.42	2.89	1.54	1.91
DL-Tryptophan	1.02	M	15.4	2.26	486	347	3.44	3.08	1.52	1.91
DL-Leucine	1.31	M	15.0	1.35	531	293	3.75	2.07	1.52	1.52
L-Arginine·HCl	0.53	M	2.44	2.26	537	347	3.83	3.08	1.52	1.91
DL-Valine	1.17	M	58.3	1.35	565	460	3.80	3.14	1.49	1.45
DL-Aspartic	1.33	M	14.5	4.02	538	352	3.64	2.80	1.46	1.71
DL-Glutamic	1.47	M	8.35	1.69	633	470	4.03	3.35	1.37	1.52
L-Serine	0.52	M	6.65	4.02	540	352	3.06	2.80	1.22	1.71
L-Proline	1.15	M	13.7	1.35	536	293	2.94	2.07	1.18	1.52
Sarcosine	0.89	M	11.8	0.75	569	372	2.95	2.28	1.13	1.31
DL-Alanine	0.89	M	12.4	1.25	537	379	2.60	3.11	1.03	1.67
Glycine	0.75	M	2.38	1.69	619	470	2.70	3.35	0.94	1.52
Creatinine	0.38	M	1.93	0.75	484	372	2.23	2.28	0.99	1.31

DL-serine by stomach tube has been observed previously by Fishman and Artom, ('42): 100 mg of DL-serine/rat/day was often fatal after three to 7 days in male rats fed synthetic diets whereas female rats were not affected; high levels of vitamin B_6 reduced the toxicity (Fishman and Artom, '44). In our experiments no deaths in either sex resulted from the large single doses of DL-serine administered, and protein excretion was increased to about the same extent in both sexes. Addition of 100 mg of pyridoxine/kg of diet reduced the protein excretion due to DL-serine to 4.16 mg of protein N/rat/day whereas omission of B_6 from the diet or the addition of 25 mg of desoxypyridoxine/kg of diet did not appear to elevate further the protein excretion, the respective values being 10.08 and 11.57 mg of protein N/rat/day. However some deaths did occur in the latter two groups following the administration of DL-serine. Protein excretion was similar; 11.45 mg and 12.53 mg of protein N/rat/day whether 0.01 moles of DL-serine or 0.005 moles of D-serine, respectively, were administered. Enhanced protein excretion therefore appeared to be due primarily to D-serine, a conclusion also reached by Artom et al. in 1945. An inability to either metabolize or reabsorb large doses of D-serine is suggested by the greater α-amino N excretion by rats given 0.005 moles of D-serine than by those given equivalent amounts of L-serine, 14.3 and 6.65 mg/rat/day, respectively. In another experiment the amount of urinary protein remained elevated for several days following the administration of a single dose of DL-serine, the successive daily values being 14.8, 7.90, 4.89 and 2.98 mg of protein N/rat/day. The excretion of α-amino N also was elevated during these same days, being 36.8, 18.7, 9.78 and 4.87 mg/rat/day, respectively.

Urea excretion by male rats given DL-serine or D-serine was markedly lower than controls, whereas that of rats receiving L-serine showed an increase similar to that observed with all of the other amino acids. This suggested that an alteration in kidney function produced by D-serine, but not by L-serine, may be responsible for the increase in protein excretion. Morehead et al. ('45) observed tubular degeneration in rats given DL-serine, and Wachstein ('47) has reported the damage to be localized in the distal region of the proximal tubule. L-Cysteine·HCl increased urinary protein by about 200% with considerable variation; rats which showed these elevations were hematuric and died within one or two days.

The other nitrogen sources tested did not alter proteinuria as markedly (table 1). The protein/urea ratios (protein N × 10^3/urea N) for the control groups varied from 1.31 to 1.91 and 12 of the amino acids tested produced protein/urea ratios within this range, indicating that they increased protein excretion essentially in proportion to their ability to produce urea. Urinary protein/urea ratios below the range of the control values resulted when L-serine, L-proline, sarcosine, DL-alanine, creatinine or glycine were administered. L-Histidine·HCl, and L-lysine·HCl produced urinary protein/urea ratios marginally in excess of those obtained for control animals.

Hardy and Baumann ('59) have suggested that the suppressing effect of glycine on proteinuria may be due to oxalic acid formed by the action of glycine oxidase. This enzyme also attacks sarcosine (Ratner et al., '44), though less effectively than glycine. Thus, the weaker role of sarcosine against proteinuria is in agreement with its lower ability to be metabolized to oxalic acid.

Urinary α-amino N showed no general relationship to the action of the individual amino acids on protein excretion. Of the amino acids which produced α-amino nitrogen excretions above 10.0 mg/rat/day, some markedly enhanced protein excretion, some produced normal elevations and others depressed it.

Urinary protein distribution by paper electrophoresis. Average mobilities of the 4 plasma peaks obtained by paper electrophoretic separation were −6.3, −4.0, −1.5 and 1.0 cm/350 volts/4 hours; for normal urine they were −6.5, −3.5 and −1.1 cm. These urinary peaks thus corresponded to the albumin, α-globulin,

TABLE 2

Paper electrophoretic distribution of urinary proteins of rats given various amino acids

Amino acids	Sex	Albumin	α-Globulins	β-Globulins	γ-Globulins
		%	%	%	%
20% Casein	M	30.2 ± 8.2[1]	51.0 ± 6.5	19.1 ± 8.5	
	F	13.0 ± 8.2	48.0 ± 7.2	39.0 ± 6.2	
10 ml H_2O	M	29.3 ± 2.5	46.6 ± 3.5	24.1 ± 4.5	
	F	15.0 ± 6.4	49.1 ± 6.8	36.0 ± 7.2	
DL-Serine	M	48.4 ± 2.6	24.3 ± 3.2	23.1 ± 2.1	5.0 ± 2.4
	F	50.2 ± 3.4	22.9 ± 2.5	20.2 ± 3.1	6.5 ± 2.1
L-Cysteine·HCl	M	16.9 ± 6.4	53.8 ± 4.8	29.3 ± 3.9	
L-Histidine·HCl[2]	M	28.1 ± 4.2	46.5 ± 3.2	25.3 ± 4.5	
DL-Methionine[2]	M	21.8 ± 3.1	49.2 ± 3.6	29.1 ± 5.2	
DL-Isoleucine[2]	M	21.8 ± 3.1	47.9 ± 3.6	30.3 ± 5.1	
DL-Leucine[2]	M	23.7 ± 3.9	48.4 ± 6.8	27.9 ± 5.2	
DL-Glutamic[2]	M	24.9 ± 6.1	47.7 ± 4.5	27.3 ± 2.5	
DL-Alanine	M	30.6 ± 3.5	46.4 ± 5.2	23.1 ± 1.5	
DL-Glycine	M	19.8 ± 4.1	41.6 ± 3.8	38.0 ± 5.0	

[1] Standard deviation.

[2] These distributions are representative of those obtained for L-lysine·HCl, L-cystine, DL-phenylalanine, L-tyrosine, DL-threonine, DL-tryptophan, L-arginine·HCl, DL-aspartic, L-proline and DL-valine (see footnote 5, p. 438).

and β-globulin fractions of the plasma, in agreement with results of moving boundary electrophoresis by Rumsfeld and Baumann ('55) and Sellers et al. ('52).

The reproducibility of the separation is represented by the following standard deviations for the relative percentages of albumin, α-globulin, β-globulins and γ-globulins, respectively, obtained from 6 separate analyses of one sample of plasma: 53.4 ± 1.8, 13.6 ± 1.6, 14.6 ± 0.85 and 18.6 ± 2.5 and of one sample of urine: 30.6 ± 2.6, 53.1 ± 1.6 and 16.3 ± 1.3 with no γ-globulin present. These latter percentages agree very well with those obtained by Rumsfeld ('56) with a moving boundary electrophoretic technique: 30.2, 49.5 and 16.5%, respectively

The relative percentages of the proteins in urine from male rats administered 0.14 gm of N as the individual common amino acids are represented in table 2. The albumin peak varied from 48.4% with DL-serine, to 16.9% with L-cysteine·HCl; the α-globulin from 53.8% with L-cysteine·HCl to 24.3% with DL-serine; and the β-globulin from 38.0% with glycine to 17.9% with L-tyrosine.

The greatest variation from normal was produced by DL-serine where albumin became the major component, 48%; α-globulin was depressed to 24% and a small variable amount of protein with the mobility of γ-globulin appeared. Female rats normally excreted a smaller percentage of albumin and a greater percentage of β-globulin than male rats, but when given DL-serine, the proteins excreted were similar to those for the treated male rats (table 2). Of the other amino acids tested, glycine produced the most consistent deviation from normal: the percentages of α-globulins and albumins were depressed whereas those of the β-globulins were elevated. This altered distribution appeared to be somewhat analogous to that obtained for female rats fed a 20% casein diet (table 2).

The normal urinary proteins of male rats on a 20% casein diet and the abnormal proteins produced by DL-serine were stained with Sudan black to detect lipid, or with basic fuchsin to detect carbohydrate. A positive stain for carbohydrate was found in the β-globulin region of both normal and abnormal urinary proteins, and it appeared to be somewhat more pronounced in the urine of rats given DL-serine. Sudanophilic material appeared in the albumin, α-globulin and β-globulin regions of both the normal and abnormal urinary proteins; the most pronounced staining occurred in the globulin regions.

Urinary protein distribution by starch-gel electrophoresis. The abnormal distribution of urinary proteins from rats given DL-serine was confirmed by starch-gel electrophoresis. Representative separa-

tions of rat plasma, normal male rat urinary proteins and abnormal urinary proteins from rats given DL-serine are presented elsewhere.[9] Normal male urine contained only three major peaks which, on the basis of their mobilities, corresponded to the albumin, α_2-globulin and β-globulin peaks of plasma. A small but variable band was found to remain at the point of application. The average percentage distribution of the proteins in the peaks from three runs were: albumin 31.5%, α_2-globulin 36.1%, β-globulins 21.3% and material remaining at the starting point 10.0%. Administration of 1.05 gm of DL-serine/rat resulted in urinary protein with peaks for albumin, α_1-globulin, β-globulin and γ-globulin. α_1-Globulin usually appeared as a "shoulder" of the albumin peak and some material also appeared to remain at the point of application. The average percentage distributions of the proteins in these peaks were: albumin and α_1-globulin 65.2%, α_2-globulin 18.0%, β-globulin 12.3%, material at the starting point 2.1%, and γ-globulin 2.4% for male rats; and albumin and α_1-globulin 60.0%, α_2-globulins 11.4%, β-globulin 18.1%, starting point material 2.8 and γ-globulin 7.6% for female rats.

Chromatographic separation of plasma and urinary proteins. The concentrated

[9] See footnote 5, p. 438.

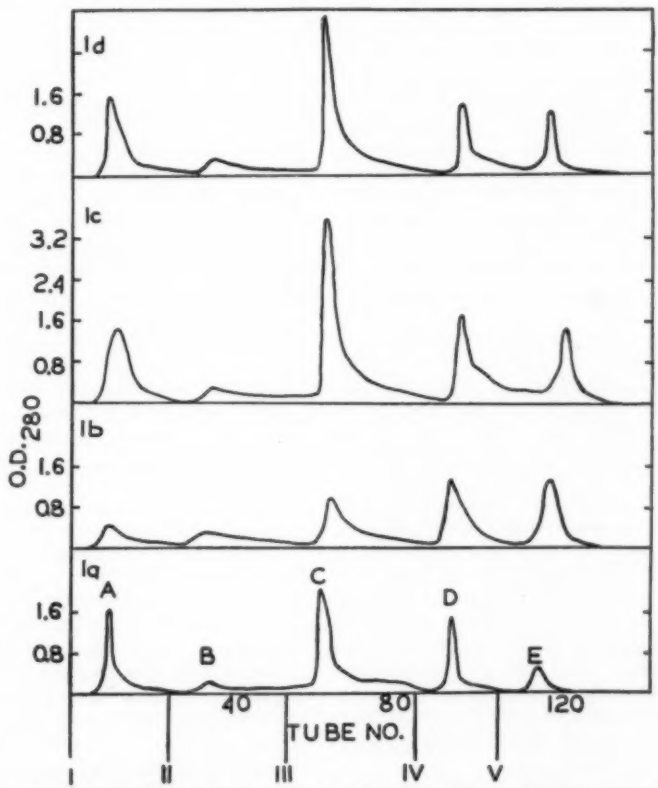

Fig. 1 Separation of proteins of (1a), male rat plasma; (1b) normal male rat urine; (1c) DL-serine male rat urine; (1d) DL-serine female rat urine by stepwise elution from DEAE-cellulose with different sodium phosphate buffers (I. 0.0175 M, pH 6.3; II. 0.04 M, pH 5.9; III. 0.10 M, pH 5.8; IV. 0.40 M, pH 5.2; and V. 0.40 M, pH 4.4 + 2.0 M sodium chloride).

TABLE 3

Relative amounts[1] of protein in chromatographic fractions from rat plasma and urine

	Peak A	Peak B	Peak C	Peak D
	%	%	%	%
Normal rat plasma	26	12	44	18
Normal male urine	17	21	33	29
Urine from male given DL-serine	21	11	43	25
Urine from female given DL-serine	28	10	43	18
Designation[2]	γ-globulin	β-globulin	albumin	α-globulin

[1] Percentage of total area for peaks A–D.

[2] Sober and Peterson ('58). Paper electrophoresis of our plasma fractions indicated the presence of β-globulin in peak A and of α-globulin in peak C.

urinary proteins prepared as described for paper electrophoresis were equalibrated against pH 6.3, 0.0175 M sodium phosphate prior to being placed on a 3.2 cm × 10 cm column of DEAE[10] which had been separately equilibrated with the buffer. The column contained 15 gm of DEAE from which the fine material remaining suspended in solution for 30 minutes had been discarded. The average flow rate of the packed column under gravity was approximately 3 ml/minute. A step-wise elution was carried out at room temperature with the sequence of buffers described by Sober and Peterson ('58) (fig. 1); 8.0 ml fractions were collected and the protein concentration determined as the optical density of the effluent at 280 mμ.

Under these conditions the proteins of urine and plasma separated into 5 distinct major peaks (fig. 1). The relative amounts of protein (percentage of total area under peaks A-D[11]) were 25, 12, 44 and 18%, respectively, for fractions A, B, C, and D of rat plasma, and 17, 21, 33 and 29% for normal rat urine (table 3); in other words, the urine contained relatively less albumin and γ-globulin than the blood. When 1.04 gm of DL-serine/rat were administered by stomach tube, the proportions of the various proteins in the urine closely resembled those of blood plasma (table 3) and this was particularly true for urine from female rats. The results of chromatography therefore substantiated the conclusions based on electrophoresis (table 2).

DISCUSSION

All except three of the amino acids tested increased proteinuria, thus supporting the general conclusion that rat proteinuria increases with nitrogen intake, whether in the form of proteins (Addis et al., '26; Linkswiler et al., '52; Rumsfeld and Baumann, '55; and Rumsfeld, '56), of urea (Finlayson and Baumann, '56) or of an amino acid mixture equivalent to casein (Finlayson and Baumann, '56). The central role of urea in proteinuria has also been confirmed, since most of the amino acids administered produced urinary protein/urea ratios within the range of the sham-treated controls. The marked exceptions to the general rule were DL-serine, D-serine, and L-cysteine·HCl, which enhanced proteinuria abnormally, and glycine and alanine which depressed it.

Amino acids which produced protein/urea ratios above or below the control range, presumably act on the kidney either by altering the amount of protein filtered or the amount of protein reabsorbed. Since the histological damage in DL-serine toxicity is a tubular degeneration (Morehead et al., '45; Wachstein, '47), decreased reabsorption of filtered protein would seem to be a major factor in this type of enhanced proteinuria. Wachstein ('47) has suggested that DL-serine might be a valuable tool for the production of a localized tubular injury because of the restricted area and the regularity of the damage to the distal region of the proximal convoluted tubules. It is within this latter region that reab-

[10] Selectacel, DEAE, Reagent grade, Brown Company, Berlin, New Hampshire.

[11] Peak E appears to be largely non-protein material, since it was not precipitated by 5% of trichloroacetic acid or 1% of phosphotungstic acid, nor did it produce a positive biuret response.

sorption of macromolecules has been shown to occur in amphibians (Gerard, '36). If one can assume that D-serine produces no damage in any other part of the kidney, and that the major site of reabsorption of protein by the tubules of the rat is analogous to that of amphibia, the markedly increased quantities of protein excreted by serine-treated rats might approach the quantities of protein that are filtered and reabsorbed in the normal rat. Sellers et al. ('54) measured the disappearance of protein-bound dye from plasma and its appearance in the tubule and concluded that protein reabsorption by the cells of the proximal convoluted tubules of the rat kidney proceeds at a rate of at least 5 mg/hour. Our results with D-serine indicated an excretion of 12.35 mg of protein N/rat/day (table 1) or the equivalent of 3.2 mg of protein ($N \times 6.25$) per hour. Since the value obtained by administration of D-serine which affects only the distal portion of the proximal convoluted tubule (Wachstein, '47) is about two thirds of the tentative value obtained by consideration of reabsorption by the whole proximal convoluted tubule, the region of the kidney affected by D-serine may be the major site of protein reabsorption and the type of proteins excreted by the serine-treated rats may then approach those of the glomerular filtrate. Comparison of the types of protein excreted by DL-serine-treated rats with those excreted by normal rats suggests the normal reabsorption of albumins and γ-globulins to be much more pronounced than that of either the α-globulins or the β-globulins.

SUMMARY

1. Rats were given large doses of single amino acids by stomach tube, and measurements were made of urinary protein, urea and α-amino nitrogen. Individual urinary proteins were determined after separation by paper or starch-gel electrophoresis, or column chromatography.

2. Most of the amino acids increased protein excretion roughly in proportion to the increased urea production. DL-Serine, D-serine and L-cysteine·HCl produced excessive elevations in protein excretion, whereas glycine and alanine depressed it.

3. Essentially similar distributions of the urinary proteins resulted from separations by paper or starch-gel electrophoresis or on a cellulose column. With respect to blood proteins, urinary proteins are low in albumin and γ-globulins and high in α- and β-globulins.

4. The administration of most of the amino acids failed to alter the distribution of the urinary proteins. However, after the administration of DL-serine the pattern approached that of plasma (albumin and γ-globulins increased). The administration of glycine to male rats yielded a pattern roughly similar to that in normal female urine (albumin and α-globulins depressed).

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Factors Affecting the Absorbability of Certain Dietary Fats in the Chick[1,2]

M. R. FEDDE, P. E. WAIBEL AND R. E. BURGER
Department of Poultry Husbandry, Institute of Agriculture, University of Minnesota, St. Paul

The presence of fat in the diet of the chicken has a beneficial influence on the efficiency of feed utilization (Sunde, '56, and others) and frequently on growth rate (Waibel, '55, and others). The chick has the ability to utilize a high proportion of its energy requirement in the form of fat (Rand et al., '58; Donaldson et al., '57).

Certain fats, however, are poorly absorbed by animals (Deuel et al., '40; Hoagland and Snider, '40). Thus, the low metabolizable energy values of animal tallow for chicks, as compared with certain vegetable oils, as found by Renner and Hill ('58), is of major importance in terms of energy conservation.

Factors which have been shown to modify the absorbability of certain fats include the age of the animal (Duckworth et al., '50; Renner and Hill, '58) and the presence of dietary mineral ions (Givens and Mendel, '17; Cheng et al., '49). The work to be reported herein compared, in the chick, the absorbabilities of certain fats and studied factors influencing the absorbability of a poorly utilized fat, namely, beef tallow. Factors studied were the age of bird, dietary addition of ox bile, restriction of feed intake and dietary calcium level.

EXPERIMENTAL

Male crossbred chicks from Dominant White ♂♂ × White Plymouth Rock ♀♀ were used in experiments 1 (10 per group) and two (7 per group), and from Vantress ♂♂ × Nichols 108 ♀♀ were used in experiments 3 (5 replicates of 5 chicks each) and 4 (4 replicates of 5 chicks each). Chicks were wing banded and placed randomly in standard electric starting batteries with raised wire-mesh floors until 4 weeks old. If the experiment proceeded longer than 4 weeks, the chicks were transferred to growing batteries.

Fat absorption trials were conducted for a three-day period during which the feed eaten and feces (total excrement including urine) excreted were quantatively measured. Wet feces were ground with the aid of an inert water absorbent.[3] Care was taken to prevent moisture loss from the feces between the time of collection and the time of analysis. The chemical analysis for fat in the feces was conducted according to the procedure described by Van de Kamer et al. ('49). The results were calculated as grams of fatty acids per 100 gm of feces. This value represents *both neutral fat and fatty acids excreted* but will be reported as fatty acids excreted, only because this is the value given by the chemical analysis. The same chemical procedure was used to determine the fat content of the feed. Apparent absorption coefficients were calculated according to the formula:

$$\frac{\text{Fat consumed} - \text{fat excreted}}{\text{Fat consumed}} \times 100.$$

Body weight and feed efficiency data were collected weekly. Feed efficiency was calculated by dividing the feed consumed by total weight gain.

Analyses of variance were used in experiments 3 and 4 to evaluate the differences, according to Snedecor ('56). If significant differences occurred, the Student-Newman-Keuls Multiple Range Test was used to locate them (Federer, '55).

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[2] From a thesis submitted by the senior author to the Graduate Faculty of the University of Minnesota in partial fulfillment of the requirements of the M.S. degree, August, 1959.

[3] Micro-Cel E, Johns-Manville, New York.

TABLE 1
Composition of diets

Ingredient	Basal	10% fat	20% fat
	%	%	%
Corn, ground yellow	63.25	45.25	26.25
Soybean oil meal, dehulled	24.75	32.75	41.75
Fat[1]	0	10.0	20.0
Constant ingredients[2]	12.0	12.0	12.0
Calculated analysis:			
Protein, %	20.76	23.31	26.29
Productive energy,[3] Cal./pound	926	1046	1161
Calorie (P.E.), % protein ratio	44.6	44.9	44.1
Calcium, %	1.24	1.27	1.29

[1] Beef tallow, hog grease, non-break safflower oil and refined corn oil were used. The value of 2760 Productive Energy Calories per pound was used for all fat sources.

[2] Constant ingredients. In per cent: corn distillers' dried solubles, 2; fish meal (60% protein), 3; alfalfa meal (dehydrated, 17% protein), 1; dried whey, 2; dicalcium phosphate, 2; calcium carbonate, 1.25; iodized salt, 0.5; vitamin A (10,000 I.U./gm), 0.03; vitamin D^3, (3000 I.U./gm), 0.02; *d*-α-tocopheryl acetate (44 I.U./gm), 0.01; choline chloride (70% soln.), 0.09; and DL-methionine, 0.05. In milligrams per kilogram of diet: chlortetracycline·HCl, 25; biotin, 0.2; menadione sodium bisulfite—63% MSB(USP), 2; pyridoxine·HCl, 2; folic acid, 2; thiamine·HCl, 2; riboflavin, 4; calcium pantothenate, 10; niacin, 20; and vitamin B_{12} (0.1% triturate), 40. In experiment 1, 0.04% manganese sulfate (feeding grade) was used. In experiment 2, 3 and 4, 0.10% of a trace mineral mixture (Delamix-Limestone Products Corporation of America, Newton, New Jersey) was used and provided the following in milligrams/kilogram of diet: manganese, 60; iodine, 1.2; iron, 20; copper, 2; zinc, 0.06; and cobalt, 0.2.

[3] Fraps, '46.

The diets used are presented in table 1.[4] The ratio of productive energy to protein was held constant, thus the only variables in the diet were corn, soybean oil meal and fat.

RESULTS

Experiment 1 was conducted to determine the apparent absorbabilities of some natural fats and oils (table 2). Essentially no differences in growth were evident among groups receiving different types and levels of fats or oils and the group receiving the basal diet. As expected, there was an improvement in feed efficiency, over the basal group regardless of the type or level of fat or oil fed.

[4] The authors wish to thank the following firms for their generous supply of experimental materials: Armour and Company, Chicago; Van Hoven Company, Inc., St. Paul, Minnesota, and Pacific Vegetable Oil Corporation, San Francisco.

TABLE 2
Utilization of dietary fats by chickens

Diets	Weights		Feed/gain		Apparent absorbability		Fatty acids excreted /100 gm feed eaten	
	4 weeks	8 weeks	4 weeks	8 weeks	1–2 weeks	7–8 weeks	1–2 weeks	7–8 weeks
	gm	*gm*			%	%	*gm*	*gm*
Basal, no added fat	441	1360	1.91	2.21	71.8	78.9	0.84	0.62
10% Safflower oil	441	1361	1.60	1.92	92.7	93.1	0.78	0.73
10% Corn oil	447	1391	1.81	2.05	85.0	90.7	1.72	1.07
10% Hog grease	425	1327	1.65	1.90	84.8	89.8	1.82	1.22
10% Beef tallow	437	1344	1.76	2.05	59.1	74.3	4.92	3.10
20% Safflower oil	429	1365	1.76	1.93	89.7	91.9	2.04	1.60
20% Corn oil	432	1426	1.63	1.98	90.2	92.4	2.01	1.98
20% Hog grease	469	1325	1.49	1.77	85.4	93.0	2.98	1.44
20% Beef tallow	452	1386	1.77	1.96	46.6	78.6	11.20	4.49
20% Beef tallow + 0.5% ox bile	424	1391	1.73	1.81	68.9	88.9	6.38	2.29

Very little fat was excreted in the feces when diets contained 10 or 20% of corn oil, safflower oil or hog grease. However, when 20% of beef tallow was fed, a large quantity of fat was excreted, especially at the early age. There was an increase in beef tallow absorbability from two to 8 weeks. The group fed 20% of tallow plus 0.5% of ox bile, absorbed the tallow more completely than the group receiving only 20% of tallow.

Experiment 2 was conducted to determine whether the increase in absorbability of beef tallow with age was due to age-mediated changes or to an adaptability of the gut (in the form of increased enzyme concentration, altered microflora, increased bile flow, and others) to the fat. Growth and feed-efficiency data are omitted since differences conformed to expectation.

Of 10 groups placed on experiment, 5 were fed the basal diet, one the 20% beef tallow diet and two the 20% safflower oil diet. Then, at two, 4, 6 and 8 weeks of age, certain groups were switched from the basal or safflower oil diets to the beef tallow diet, as shown in figure 1. Since variability was small among results of identical treatments, the data are combined into one line for the benefit of simplicity in presentation. The two other treatments in this experiment (not shown) were a 10% beef tallow-10% hog grease combination and a 20% beef tallow group switched to 10% beef tallow at 6 weeks of age.

The birds which received the basal and safflower oil diets excreted very little fat when compared with groups fed the beef tallow diets. The amount of fat excreted by the groups fed beef tallow decreased with increasing age. When 20% beef tallow diets were substituted for the basal diets, at the various ages, the amount of fat excreted was approximately the same as that excreted by the respective birds fed the 20% tallow diet continuously.

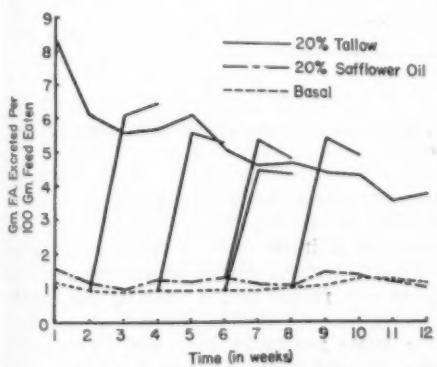

Fig. 1 Effect of age on fat absorbability. (See results section for details.)

When the 20% beef tallow diet was changed to a 10% beef tallow diet at 6 weeks, the birds exhibited no increase in apparent fat absorbability. The group which received 10% each of beef tallow and hog grease gave absorbability results intermediate to the 20% beef tallow and 20% safflower oil groups.

Experiment 3 was designed to study the influence of graded levels of ox bile on the fat absorbabilities of diets containing 20% of beef tallow (table 3). The 4 and 8% ox bile levels caused a significant growth depression throughout the experiment. Feed efficiencies paralleled growth, namely, when 4 and 8% ox bile levels were fed, feed was least efficiently utilized.

Greatly enlarged gallbladders were found in birds fed 0.5% of ox bile in experiment 1. Thus, to verify this observation, the bile in the gallbladders was removed and measured with a calibrated syringe at the end of the 4-week period in this experiment. It was assumed that the amount of bile present was indicative of the gallbladder size. Visual observations indicated that the gallbladders of birds that received high levels of ox bile were greatly enlarged when compared with the other organs, such as the liver. The results in table 3 show that gallbladders of birds fed the 8% ox bile diet were relatively larger than those of birds fed any other diet and that ox bile levels over 1% resulted in a significant increase in bile storage.

Although the apparent absorbability of the 20% beef tallow diet was higher than had been found previously, a significantly higher absorbability of fat was found when ox bile levels of 0.5% and higher were fed. No significant difference in the absorption coefficients existed between 8, 4, 0.5 and 1% ox bile levels. This suggests that 0.5% of ox bile is virtually as effective in increasing fat absorption as is the high 8% level.

TABLE 3

Effect of dietary ox bile on growth, gallbladder size and fat absorption of chicks fed 20% of beef tallow

Four-week weights:								
Ox bile level, %	0	0.1	1	2	0.5	0.05	4	8
Means,[1] gm	611	597	577	575	567	556	409	243
Ratio of CC. bile in gallbladder to body weight (in gm) × 100:								
Ox bile level, %	0	0.1	0.05	0.5	1	2	4	8
Means[1] of ratio	0.11	0.14	0.18	0.18	0.20	0.20	0.25	0.38
Apparent absorption coefficients:								
Ox bile level, %	0	0.1	0.05	2	1	0.5	4	8
Means,[1] 21–23 days, %	83	84	85	86	87	88	89	90

[1] Any two or more means not underscored by the same line are significantly different at the 0.05 level of probability. See text for methods.

TABLE 4

Effect of restricted feed intake and dietary calcium levels on growth, feed efficiency and fat absorption of chicks fed 20% of beef tallow

Four-week weights:					
Treatment	Low Ca	Control	High Ca	80% AL[1]	60% AL[1]
Means,[2] gm	539	524	480	431	336
Four-week feed efficiency:					
Treatment	High Ca	Control	80% AL[1]	60% AL[1]	Low Ca
Mean,[2] feed/gain	1.92	1.74	1.71	1.71	1.63
Apparent fat absorption coefficients:					
Treatment	Low Ca	Control	80% AL[1]	60% AL[1]	High Ca
Mean,[2] 11–14 days, %	88	71	71	71	56
Means,[2] 25–28 days, %	91	77	76	75	71

[1] Ad libitum.

[2] Any two or more means not underscored by the same line are significantly different at the 0.05 level of probability. See text for methods.

In experiment 4, the effects of restricted feed intake and dietary calcium level on growth, feed efficiency and fat absorbability by chicks were measured (table 4). The treatments used were (1) control diet, 20% of beef tallow, normal calcium (1.24%), ad libitum; (2) control diet fed at 80% ad libitum after 7 days of full feeding, the intake being calculated from the preceding day's consumption by the full-fed controls; (3) control diet fed at 60% ad libitum, carried out the same as (2); (4) control diet in which the calcium level was raised to 3% during the entire experiment by adding 45 gm of calcium carbonate per kg of diet; and (5) control diet, in which the calcium level was lowered to 0.33% during the digestion trials

(4 days, i.e., one day preceding and 3 days during trial) by removing calcium carbonate and dicalcium phosphate. The total phosphorus level was lowered to 0.49% during the periods when this diet was fed.

Birds fed restricted diets had lower body weights than the controls, in accordance with the degree of restriction. However, restricted feed intake caused no change in feed efficiency or the ability of the birds to absorb beef tallow when compared with the controls. A high 3% dietary calcium level depressed growth and feed efficiency somewhat, and also lowered the absorbability of beef tallow at two and 4 weeks of age. Growth or efficiency of feed utilization was not altered, over that of the control, by lowering the calcium level to 0.33% and the phosphorus level to 0.49% during the absorption trials. However, this did result in a striking improvement in the absorbability of beef tallow.

DISCUSSION

It was not possible, by using apparent absorption coefficient values, to compare the amount of fat excreted by birds fed high and low fat diets in experiments 1 and 2. Data in table 2 illustrate that the apparent absorbability of the fat in the basal diet, which contained approximately 3% of fat, was much lower than would be expected. The reason for this value is not low absorbability of corn and soybean oils, but is due mainly to the excretion of endogenous fecal (and urine) fat. A correction for endogenous and basal unabsorbed fat was not possible, since the amount of fat in the basal components of the diet was not constant at each level of added fat. Carver et al. ('55) used "total fatty acids excreted per 100 grams of feed eaten" to illustrate differences of fat utilization between high- and low-fat diets. This method has been used in experiments 1 and 2 of this study to show these differences. Where comparisons were made between treatments when only one fat level was used, as was done in experiments 3 and 4, apparent absorption coefficient values were very successfully used.

The absorbability of certain fats by the chick increases with age. It was postulated that if an adaptability of the gut to the fat was responsible for the increase in absorbability of beef tallow observed in experiment 1, then approximately the same large amount of fat, as shown by the very young chick, would be excreted in the feces, after the change from the low- to the high-fat diet, at either 2, 4, 6 or 8 weeks of age. However, as shown in figure 1, the amount of fat excreted, when the diets were so changed, decreased as age increased, and approximated the amount excreted by the group which received the high beef tallow diet continuously. Either the gut adapted rapidly to the fat (within the three-day adjustment period prior to collection), or the adaptability factors mentioned previously were not the reason for the increase in fat absorbability. More evidence which points to the latter alternative is provided by the group switched from the 20% beef tallow diet to the 10% beef tallow diet at 6 weeks of age. If adaptability factors were increased by feeding a diet high in fat, then simply reducing the quantity of fat fed should cause an increase in the utilization of that fat. However, this was not the case, and again the absorbability appeared to be related primarily to age.

The amount of fat excreted at one week of age, when 20% of beef tallow was fed, was very large. This was quickly reduced by the second week of age and proceeded to gradually decline. This indicates that the absorptive mechanism for certain fats in the very young bird is not well developed. Since safflower oil and corn oil are well utilized at this young age, it appears that the physiological or chemical processes involved in their digestion or absorption is, indeed, different from those involved with beef tallow.

It is possible that the exogenous ox bile, which would be in solution in the gut, has two functions. It may aid directly in fat absorption and/or it may stimulate the liver cells to secrete more bile acids which would aid in fat absorption. The gallbladder size of birds which received the higher ox bile levels was increased over that of the control birds. This evidence suggests that, although the bile acids present in the ox bile were not characteristic of the birds' bile acids (Deuel, '55), they were probably absorbed and had a choleretic effect on the liver.

The addition of bile caused a more dramatic fat absorption response in the pre-

liminary experiment (table 2) than in the comprehensive experiment (table 3). Unfortunately, another batch of beef tallow, showing fairly good absorbability, was used in the comprehensive experiment. This variability in tallows is important and requires further consideration.

The results of experiment 4, which tested the effect of restricted feed intake on the absorbability of fat, indicate that absorbability of fat is not dependent upon growth rate. Birds which were fed 80 and 60% ad libitum diets had much lower body weights than the controls but did not differ from the controls in their ability to utilize beef tallow.

Cheng et al. ('49) found that dietary calcium had a marked influence on the absorbability of fat in the rat. Our results clearly illustrate the same phenomenon with the chick. It is postulated that with a decrease in the ionized calcium level in the gut, a decrease in the formation of insoluble soaps occurred and more of the tallow could then be utilized.

Thus, in this paper, two factors, namely, bile sufficiency and level of ionized calcium, are shown separately to affect the absorbability of beef tallow. Whether their effects would be additive was not determined. Further work will be required to determine the relative importance of each in the age adaptation effect noted earlier.

Generally, results in experiment 1 indicate that the dietary level of fat (10 or 20%) does not alter utilization, in agreement with the results of Hoagland and Snider ('41) using rats, and thus one might assume that bile could not be limiting at the higher fat level. However, the utilization observation is a result of a combination of factors which may produce opposite effects on absorbability at various fat levels. For example, whereas a high-fat level would be expected to compete more severely for a limiting amount of bile than a lower fat level, it would have relatively less interference from the existing level of calcium and magnesium ions.

SUMMARY

The apparent absorption coefficients of fats by chicks were similar for either 10 or 20% dietary-fat levels and were high for safflower oil, corn oil and hog grease, and low for beef tallow.

The apparent absorbability of beef tallow (a) increased from 53% at one week of age to 80% at 12 weeks of age, (b) was increased when 0.5% or more of dietary ox bile was added, (c) was not dependent upon growth rate or feed intake and (d) was decreased by high dietary calcium and increased by reducing the calcium intake.

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Blood Pressure and Thiocyanate Space in the Vitamin B_6-Deficient Rat During Pregnancy[1,2]

MYRTLE L. BROWN[3,4] AND RUTH L. PIKE
Department of Foods and Nutrition, The Pennsylvania State University, University Park

Reports from this laboratory (Ross and Pike, '56) and another (Nelson and Evans, '51) have shown that vitamin B_6 is essential for normal reproduction in the rat. In addition to the usual criteria of maternal weight gain, litter size and weight, and incidence of resorption, other manifestations of an abnormal response to pregnancy have been observed in the deficient animal (Ross and Pike, '56; Pike and Brown, '59). Several investigators (McGanity et al., '49; Wachstein and Gudaitis, '52; Sprince et al., '51) have suggested a causative relationship between vitamin B_6-deficient rats by Olsen and nancy in the human. In view of the reported increase in blood pressure in vitamin B_6- deficient rats by Olsen and Martindale ('54), and the impairment in water metabolism suggested by the studies of Stebbins ('51) and Guggenheim ('54), we were interested in studying changes in the pregnant animal which might be related to toxemia of pregnancy. The present report deals with weekly changes in blood pressure, thiocyanate space and specific gravity of vitamin B_6-deficient and control animals. In order to separate the changes due to the deficiency from those due to pregnancy, observations were made also on nonpregnant animals under similar conditions.

EXPERIMENTAL PROCEDURE

Young adult female albino rats of the Sprague Dawley strain were maintained on laboratory chow until they had reached a weight of approximately 200 gm and regular estrous cycles had been established. Estrous cycles were followed by means of daily vaginal smears. Two groups of animals were subjected to a depletion period on the basal diet (table 1) containing 2.0 mg% of deoxypyridoxine for a minimum of 6 days prior to mating.[5] On the day that mating was confirmed by the presence of sperm in the vaginal smear, one group was continued on the basal diet supplemented with 2.0 mg% of deoxypyridoxine and the other received the basal diet supplemented with 0.8 mg% of pyridoxine. A control group of animals was maintained on laboratory chow until the day of mating and was given the diet containing 0.8 mg% of pyridoxine for the three-week gestation period. In addition, three groups of nonpregnant animals corresponding to each of the pregnant groups were maintained under similar experimental conditions. Feeding was ad libitum and individual records of food consumption were kept for each week of the experimental period.

Systolic blood pressure was determined using a photoelectric tensometer as described by Kersten et al. ('47). Six consecutive readings were taken and averaged for each animal. Blood pressure determinations were always made by the same operator in order to avoid the possibility of variation in technique.

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[3] Taken in part from a dissertation submitted to The Graduate School of The Pennsylvania State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[4] Mary Swartz Rose Fellow, 1955–56. Present address: Human Nutrition Research Division, Agricultural Research Service, U. S. D. A., Washington, D.C.

[5] The authors wish to express their appreciation to Merck Sharp and Dohme for their kindness in supplying vitamins for this study.

TABLE 1

Basal diet

Constituent	Amount	Vitamin mixture[1]	Amount
	%		*mg*
Casein, vitamin test[2]	26	thiamine	2.0
Sucrose and vitamin mixture	18.45	riboflavin	2.0
Cornstarch	34	*p*-aminobenzoic acid	200.0
Hydrogenated fat[3]	10	niacin	10.0
Corn oil[4]	5	pantothenic acid	8.0
Salt mixture[5]	4	biotin	0.04
Agar	2	inositol	400.0
Choline	0.4	vitamin B_{12} triturate[6]	4.0
L-Cystine	0.15	folic acid	0.4
Vitamin A, D, and E mixture[7]		naphthoquinone	1.0

[1] Made up to 18.45 gm with sucrose.
[2] Labco, The Borden Co.
[3] Crisco, Procter and Gamble.
[4] Mazola, Corn Products Refining Co.
[5] (Hawk and Oser, '31.)
[6] A 0.1% trituration of crystalline B_{12} in mannitol (Merck).
[7] Vitamin A, D, and E mixed in corn oil contained 5,000 I.U. of A, 400 I.U. of D_3 and 10 mg of α-tocopherol in two drops and was administered two drops per rat every three days.

Thiocyanate space was determined by an ultra micro adaptation of the method of Eder ('51) using 5 μl of serum. Animals were deprived of food for three hours prior to the determination. An injection of 0.1 ml of 5% sodium thiocyanate[6] was given into a caudal vein. Blood samples for analysis were taken from the tip of the tail both prior to and 20 minutes after the injection. Preliminary tests indicated that equilibration occurred at 20 minutes following the injection of thiocyanate. This technique has been used by Aikawa ('50) in the rabbit and is advantageous in that it eliminates frequent bleeding of the animal in order to extrapolate to zero time. In all, not more than 0.1 ml of blood was taken from an animal.

As an additional indication of body composition, specific gravity of the live animal was determined by a method developed in this laboratory (Pike and Brown, '53).[7]

All tests were done on the first, 8th, 15th and 21st days of experiment. The data were analyzed statistically by means of an analysis of variance to determine the effects due to pregnancy, diet and depletion. Corrections were made for disproportionality among the groups.

RESULTS AND DISCUSSION

Blood pressures obtained in this study are shown in table 2. These results agree with the reported range of 100 to 123 mm of mercury for normal rats, 3 to 4 months old and weighing 200 to 300 gm (Heymann and Salehar, '49). In none of the deficient or pregnant animals was an increase in blood pressure observed.

These results are not in agreement with those reported by Olsen and Martindale ('54). The reason for the discrepancy between the two studies is difficult to assess.

[6] Solution also contained 0.5% of Evans blue dye for blood volume determinations. Blood volume data will be reported in a later publication.

[7] Pike, R. L., and W. N. Brown, Jr. 1953 Federation Proc., *12*: 426 (abstract). For specific gravity determination, the equipment required is a battery jar containing water at approximately 37°C, a nylon net bag with weight attached and a length of nylon thread with two hooks approximately one foot apart. The thread was suspended from a Toledo scale so that the hooks were just above the water level. The animal was weighed and then placed in the bag and the open end of the bag was sewed loosely with nylon thread. The bag was attached to the upper hook so that only the weight on the bag was immersed in water, and the weight of the animal in air was obtained. The bag containing the animal was then attached to the lower hook. The animal was immersed in water and the weight was obtained quickly. The weight attached to the bag prevented the animal from floating and did not figure in the calculation since it was weighed under water both times. Volume was calculated by subtracting the weight of the animal in water from the weight in air. Specific gravity was calculated by dividing the actual weight of the animal by its volume.

TABLE 2

Average blood pressure (in millimeters of mercury)

	Nonpregnant				Pregnant			
Day of experiment	1	8	15	21	1	8	15	21
Nondepleted								
0.8 mg% Pyridoxine	108 ± 3[1]	110 ± 2	109 ± 2	109 ± 1(7)[2]	108 ± 2	109 ± 4	110 ± 2	109 ± 1(7)
Depleted								
0.8 mg% Pyridoxine	109 ± 3	108 ± 4	110 ± 2	107 ± 2(11)	108 ± 2	108 ± 2	108 ± 2	107 ± 3(8)
2.0 mg% Deoxypyridoxine	107 ± 5	107 ± 5	104 ± 4	101 ± 6(9)	108 ± 2	109 ± 3	107 ± 3	107 ± 3(8)

[1] Standard error of the mean.
[2] Number of animals in group.

It has been established that young animals are more easily depleted of their vitamin B_6 reserves than older animals (Miller and Baumann, '44). In this study animals were placed on the deficient diet at a later age than those of Olsen and Martindale. However, the level of deoxypyridoxine used was sufficiently high to produce a severe deficiency as evidenced by loss of weight and the appearance of acrodynia and alopecia. It appears probable that the reported increase in blood pressure must be attributed to more than a vitamin B_6 deficiency per se. Hsu et al. ('58) have reported an increase in serum sodium and a concomitant decrease in potassium in the deoxypyridoxine-fed rat and suggest that the elevation of serum sodium may bear a causative relationship to the increase in blood pressure observed by Olsen and Martindale ('54). The present study indicates that elevated blood pressure does not occur under all conditions of vitamin B_6 deficiency. It is suggested that vitamin B_6 deficiency may lead to fundamental physiological or metabolic changes which, under certain conditions, may be reflected in changes in electrolyte balance or in blood pressure but not necessarily in both. Some aspects of this problem are currently being investigated in this laboratory.

Data for total thiocyanate space and for thiocyanate space expressed as percentage of body weight are shown in figure 1. Only 4 of the 8 animals in the pregnant group maintained on deoxypyridoxine were able to implant and carry young to term following successful mating. Those that carried young were maintained on the depletion diet for an average of 6 days before mating and gained 18 gm during the gestation period. The other animals were maintained on the diet for an average of 13 days and lost 19 gm during their unsuccessful pregnancies. The longer depletion period in these animals was due to the failure to mate on early trials and to irregular estrous cycles observed in the depleted animals. Data for these animals, therefore, are shown as an average for the group and as averages for the animals with and without litters.

Significant increases in total thiocyanate space occurred in pregnant rats fed the

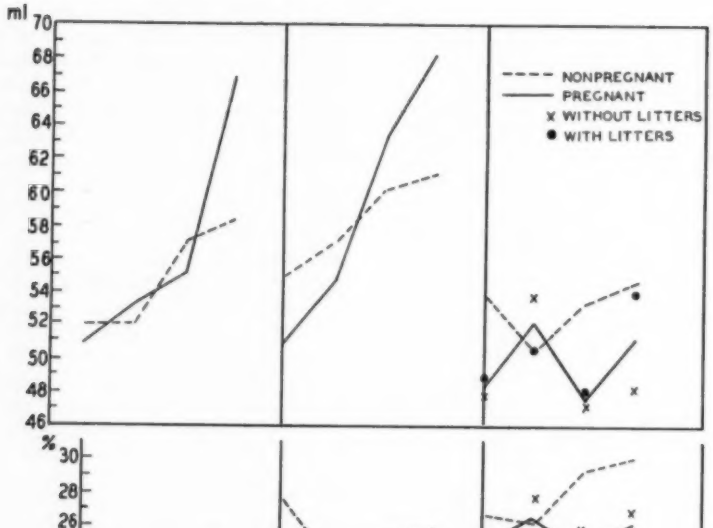

Fig. 1 Average thiocyanate space for pregnant and nonpregnant rats (in milliliters and in percentage of body weight).

pyridoxine-containing diet ($P = 0.01$). These increases reflect the normal hydration of pregnancy. Increases in total body water in the pregnant rat on the basis of chemical analyses have been reported by Spray ('50) and Beaton et al. ('53). Since gain in body weight was somewhat greater than gain in total thiocyanate space, the percentage of thiocyanate space decreased slightly as pregnancy progressed.

Nonpregnant animals fed the pyridoxine-containing diet gained weight during the experimental period and an accompanying increase in total thiocyanate space was observed. The percentage of thiocyanate space remained essentially constant suggesting that relative body composition was unchanged.

Significant increases in total thiocyanate space ($P = 0.001$) were observed on the 8th and 15th days in depleted animals fed the pyridoxine-containing diets. This reflects the immediate gain in weight when animals are shifted from a deficient diet to one containing the vitamin. Total and percentage of thiocyanate space were similar at the end of the experimental period in nondepleted and depleted animals fed pyridoxine and it appears, therefore, that the effect of prior depletion on thiocyanate space was offset in both pregnant and nonpregnant animals by the diet containing 0.8 mg% of pyridoxine.

Only slight increases in total thiocyanate space were observed in pregnant animals fed deoxypyridoxine. Levels of total thiocyanate space were somewhat higher in animals carrying young than in those in which pregnancy terminated early in the gestation period, but this difference was not significant. It appears that the deficient animals attempted to make the nor-

TABLE 3

Average specific gravity

Day of experiment	Nonpregnant		Pregnant	
	1	21	1	21
Nondepleted				
0.8 mg% Pyridoxine	0.965 ± 0.021[1]	0.970 ± 0.020(7)[2]	0.968 ± 0.010	0.994 ± 0.013(7)
Depleted				
0.8 mg% Pyridoxine	0.966 ± 0.019	0.978 ± 0.017(11)	0.984 ± 0.012	0.994 ± 0.010(8)
2.0 mg% Deoxypyridoxine	0.952 ± 0.034	0.964 ± 0.028(9)	0.963 ± 0.015	0.971 ± 0.020(8)

[1] Standard error of the mean.
[2] Number of animals in group.

mal response to pregnancy but could not overcome the effects of the deficiency. The percentage of thiocyanate space tended to be somewhat larger in these animals than in the pyridoxine-fed pregnant animals but the difference was not significant.

The trend for the nonpregnant animals fed deoxypyridoxine was similar to the pregnant deficient animals, but more pronounced. These animals lost weight while maintaining fairly constant levels of total thiocyanate space. As a result, thiocyanate space increased significantly ($P = 0.001$) in relation to body weight. No visible evidence of general edema was found in these animals nor were the levels of percentage of thiocyanate space of the magnitude at which edema has been observed in the rat (Meneely et al., '53). The relative increase in percentage of thiocyanate space in this study might be a reflection of loss of body fat. This interpretation is consistent with the findings of Guggenheim and Diamant ('59). Increases in the percentage of thiocyanate space were greater in the nonpregnant deficient group than in the pregnant deficient group. The nonpregnant animals unavoidably were kept on the depletion diet for approximately 8 days longer than the pregnant group. The greater increase in the percentage of thiocyanate space may, therefore, be a result of the more severe deficiency produced in the nonpregnant animals or a deficiency uncomplicated by the changes occurring during pregnancy. Since no intensification of the effect was observed in the pregnant animal, these data indicate that the increase in the percentage of thiocyanate space is due chiefly to the deficiency and appears to be unaffected by the additional stress of pregnancy.

Olsen and Martindale ('54) failed to find a significant difference between extracellular space of vitamin B_6-deficient and control animals using relative levels of radiosodium distribution as the criterion. Their analyses were made rather early in the experimental period. Possibly the differences might have been found if the determinations had been made when the animals had been maintained on the diet for a longer time.

Attempts to correlate either total or percentage of thiocyanate space with specific gravity of the animals were, in general, disappointing. Specific gravity as determined by this method is a relative measure of body composition. Thus, it is the change in specific gravity which serves as an index of change in body composition. Good agreement was noted between thiocyanate space and specific gravity for the nondepleted pregnant controls, but in no other group. The reason for this is difficult to understand. It may be that when body composition is relatively constant, as seemed to be the case for the nonpregnant controls, or where changes in body composition are complicated by the effects of depletion, as in the other groups, the specific gravity method is not precise enough to serve as a measure of body composition.

SUMMARY AND CONCLUSIONS

Changes in blood pressure and thiocyanate space have been investigated in

pregnant and nonpregnant vitamin B_6-deficient and control rats.

Blood pressures were within the normal range. No evidence was observed of hypertension as a result of vitamin B_6 deficiency in either pregnant or nonpregnant rats under the conditions of this study.

Total thiocyanate space increased significantly in pregnant animals fed pyridoxine and to a lesser degree in pregnant animals fed deoxypyridoxine. When calculated on a basis of body weight, thiocyanate space increased in deficient animals indicating a tendency toward water retention in vitamin B_6 deficiency. This effect appeared to be due chiefly to the deficiency and was not intensified by the additional stress of pregnancy. While the relative increase in the percentage of thiocyanate space during vitamin B_6 deficiency may be associated in part with loss of body fat, it is conceivable that this tendency toward water retention, though not intensified by pregnancy, might be related to the water retention observed in toxemias. Some aspects of this problem are currently being investigated in this laboratory.

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Effects of Ethionine on Neonatally Castrated Male Rats[1]

JEROME A. GRUNT,[2] JAMES T. HIGGINS, JR.[3] AND CHARLES B. HAMMOND

Departments of Anatomy and Pediatrics, Duke University School of Medicine, Durham, North Carolina

Aged, neonatally castrated male rats of a locally inbred Osborne-Mendel strain develop significantly less hepatic fat than do litter-mates castrated as adults (Grunt et al., '58). Other authors have shown that ethionine treatment of rats castrated as adults leads to accumulation of large amounts of hepatic fat. This was attributed to a relative deficiency of methionine due to a competitive inhibition by ethionine (Farber et al., '51). The present study was carried out to determine whether ethionine causes different degrees of hepatic fatty metamorphosis in adult male rats castrated either at birth or as adults. In order to analyze these potential differences, studies of hepatic structure and function have been carried out.

EXPERIMENTAL

Male rats of a locally inbred derivative of the Osborne-Mendel strain, mentioned above, were used. This colony was established at Duke University in 1939, from stock obtained from Vanderbilt University. Brother and sister mating has been used since the colony was established.

For the purpose of brevity, the following definitions have been used: *adult castrates*, adult male rats castrated as adults; *neonatal castrates*, adult male rats castrated at birth; *adult, intact animals*, adult male rats which have not been castrated; *gonadal status*, the presence or absence of gonads (testes) and the age at which castration occurred.

Fifty animals were divided into three groups as follows: group 1, 16 adult intact animals, 77 to 100 days old; group 2, 18 adult castrated animals, 81 to 125 days old, which had been castrated at 53 to 98 days of age and sacrificed 28 days after castration; group 3, 16 adult rats, neonatally castrated, 77 to 255 days of age. Litter-mates were used where possible. All animals were caged in the same manner and fed a commercial chow[4] and water ad libitum.

All animals were fasted 24 hours prior to injection. One half of each of the three groups was given intraperitoneal ethionine, 1 mg/gm of body weight. The ethionine concentration used was 25 mg/cm³. The dosage was divided into three equal parts given two and one-half hours apart. The remaining one half of each group of rats was injected with an equivalent amount of distilled water using the same time interval between injections. All animals were anesthetized with ether and exsanguinated from the abdominal aorta 24 hours after the first injection.

At autopsy, livers were removed, weighed and a sample of approximately 50 mg was taken for routine and fat-stained sections. The livers were dried for 72 hours at 110°C and the fat extracted with petroleum benzin. All histologic sections were studied by at least two observers who were unaware of animal treatment.

The following liver function tests were carried out upon the rat serum:

1. Bilirubin (Natelson, '51; Van den Bergh et al., '13).

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[4] Purina Fox Chow, Ralston Purina Co., St. Louis, Mo.

2. Glutamic-pyruvic transaminase[5] (Karmen, '55).

3. Alkaline-phosphatase[6] (Bessey et al., '46; Fujita, '39).

4. Thymol turbidity (Natelson, '51; Maclagan, '44; Shank et al., '46).

5. Serum total protein (Reiner, '53; Hiller et al., '48).

6. Electrophoresis of serum proteins.[7]

RESULTS

At autopsy, ethionine-treated animals castrated as adults and animals castrated at birth had large, pale, yellow-brown mottled livers. A slight amount of this mottling was seen in the livers of the ethionine-treated adult, intact animals. The livers of the animals in all groups treated with water were essentially normal to gross observation.

Fat extraction yielded results which agreed with gross observations. In the neonatally-castrated and adult-castrated groups treated with ethionine, no essential differences were found in the concentrations of hepatic fat. The amount of hepatic fat in both castrated groups was, however, significantly greater than the amounts contained in the ethionine-treated intact adult group. Figure 1 shows the relative amounts of hepatic fat per gram of liver.

No statistically significant difference was noted in any of the groups, regardless of treatment, relative to wet-liver weight (mean of all groups, 5.86 gm), or hepatic water as percentage of wet-liver weight (mean of all groups, 71.0%). Table 1 lists the animal weights, ages and hepatic fat content.

The histologic sections of all livers paralleled the gross and analytical findings. Sections from livers of animals treated with ethionine showed massive periportal fatty metamorphosis, loss of normal architecture with cytomegaly, vacuolization, "foamy" cytoplasm and passive congestion. Liver sections from all animals given water were essentially normal to microscopic observation.

Liver function tests

1. *Bilirubin.* In comparison with all groups treated with water, a significant increase was observed in the direct Van den Bergh reaction in the ethionine-treated groups. The most marked increase was in the ethionine-treated neonatally-castrated group. The indirect Van den Bergh values were decreased in all ethionine-treated ani-

[5] Sigma Chemical Company 1958 A Method for Determining Serum Glutamic Pyruvic Transaminase. Technical bull. 505.

[6] Sigma Chemical Company 1958 A Method for Determining Alkaline-Phosphatase in Serum. Technical bull. 104.

[7] Beckman Instruments Inc., Spinco Division 1959 Instruction Manual, Model R Paper Electrophoresis System-RIM-5. Technical bull. 6026A.

TABLE 1

Effects of ethionine on liver fat of neonatally castrated male rats

Treatment	No. of animals	Mean weight of animals	Mean age	Mean weight of liver
		gm	*days*	*mg fat/gm*
Intact adult + H_2O	8	202–279 (242)	77–97 (86.8)	4.8–23.7 (11.7 ± .08[1])
Intact adult + ethionine	8	200–272 (245)	85–100 (91.1)	11.6–109.4 (38.1 ± .38)
Adult castrate + H_2O	9	198–293 (242.7)	81–125 (110.5)	6.00–30.6 (18.6 ± .08)
Adult castrate + ethionine	9	212–312 (265.9)	95–121 (111.2)	47.7–122.0 (69.9 ± .31)
Neonatal castrate + H_2O	8	168–298 (238.1)	75–255 (131.0)	2.2–21.2 (9.5 ± .09)
Neonatal castrate + ethionine	8	188–296 (229)	95–255 (144.9)	11.9–181.6 (74.8 ± .70)

[1] Standard error of the mean.

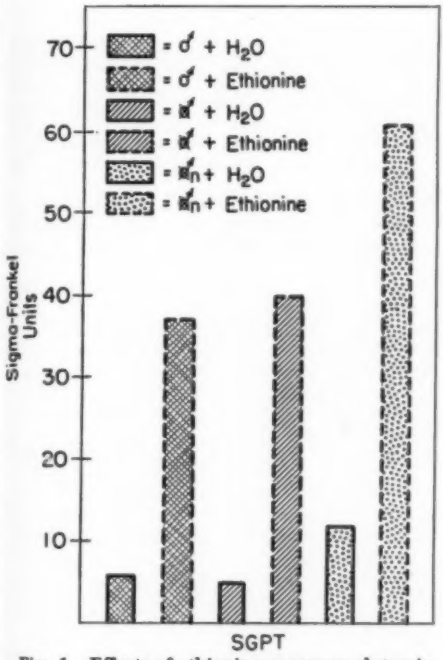

Fig. 1 Effects of ethionine on serum glutamic-pyruvic transaminase in intact and castrated male rats.

mals. No patterns of significant change were apparent in the total bilirubin determinations, regardless of treatment or gonadal status.

2. *Serum glutamic-pyruvic transaminase* (SGPT) (*fig. 1*). The SGPT levels of all groups treated with ethionine were markedly increased when compared to SGPT values of water treated animals. Highest SGPT values were found in the ethionine-treated neonatal-castrated group. The SGPT values of ethionine-treated animals castrated either as adults or at birth were significantly increased above water treated animals of similar gonadal status.

3. *Alkaline-phosphatase and thymol turbidity.* No significant changes occurred in any of the groups, regardless of treatment, in either the alkaline-phosphatase or thymol turbidity tests.

4. *Serum protein and electrophoresis.* No significant change was noted in the total protein determination in any group, regardless of treatment. The only change in the protein fractions was a slight decrease in the α-2-globulin in all ethionine-treated groups as compared with comparable water-treated groups.[8]

DISCUSSION

Much research has been carried out in relation to nutritional hepatic injury, and in demonstrating the importance of the sulfur amino acids (György et al., '39; Glynn, '45). This work has been stressed recently by Farber[9] through the demonstration of the effects of ethionine on the liver. Ethionine is an analogue of methionine and is thereby a possible inhibitor of protein synthesis (Farber et al., '50).

Contrary to the findings of Farber et al. ('51) this study demonstrated that hepatic fat was significantly increased in ethionine-treated adult, intact animals, when compared with water-treated animals of the same gonadal status. Since the methods of ethionine injection and dietary control were similar to those used by Farber, this difference may be due to an increased susceptibility to the effects of ethionine in the Osborne-Mendel strain of rats used in this experiment.

Sidransky and Farber ('58) demonstrated similar periportal hepatic fatty metamorphosis in female rats force-fed methionine-devoid diets, and female rats injected with ethionine. In male rats injected with ethionine or force-fed methionine-deficient diets, no periportal fatty metamorphosis was noted. This evidence lends further support to the theory that ethionine exerts some of its effects by interfering with the metabolic role of methionine. It also demonstrates the previously mentioned "protective" effect of androgens.

Ethionine-treated rats castrated as adults developed significantly more hepatic fat than ethionine-treated adult, intact animals (Farber et al., '51). Other workers have shown that in the castrated

[8] Specific values for all of the aforementioned tests are available to interested persons. These may be obtained by requesting them from the authors.

[9] Farber, E. 1955 Relationship of disturbed hepatic protein metabolism to fatty liver induced by ethionine. Federation Proc., *14*: 402, (abstract).

diet: thiamine·HCl, 0.25; riboflavin, 0.5; pyridoxine·HCl, 0.25; Ca pantothenate, 2.0; nicotinic acid, 1.0; choline chloride, 100.0; biotin, 0.01; folic acid, 0.1; inositol, 10.0; 2-methyl-1,4,-naphthoquinone, 0.1; and cyanocobalamin (vitamin B_{12}), 0.01. The salt mixture was that described by Hegsted et al., ('41). Animals assigned to the deficient diets received the basal ration devoid of a single essential amino acid, either valine or lysine. Sucrose was substituted for the missing amino acid. Each ration was blended with water so that each milliliter of diet contained from 0.8 to 1 gm of diet. The resulting mixture was in a suitable form for administration by stomach tube.

In the force-feeding experiments animals were divided into three groups according to diet: complete (control), valine-devoid, and lysine-devoid. Force-feeding was performed according to the method of Shay and Gruenstein ('46) using plastic tubes. The rats were fasted overnight before the special diets were started for three to 6 days. The complete ration was force-fed to the control rats throughout and to all experimental animals one day prior to beginning the deficient diets. The ration was given twice daily, at 9 A.M. and 5 P.M. The rats received an average daily feeding of 0.8 gm of ration per 10 gm of initial body weight. After being fed for three, 5, or 6 days, rats were anesthetized with ether and exsanguinated in the morning, approximately 18 hours after the last feeding. The findings were identical in animals force-fed the experimental diets for 5 and 6 days. Therefore, in reporting the results, the two groups have been combined.

In the ad libitum experiments the animals were divided into three groups in a similar manner as in the force-feeding experiments: complete (control), valine-devoid, and lysine-devoid. The rats were fasted overnight before starting to receive the special diets for 7 days. Rats had available at all times the liquid diet mixture composed of a concentration of 0.5 to 0.8 gm/ml. Diet was available until the morning the animals were killed.

Rats were weighed at the beginning and at the termination of each experiment. The organs were weighed fresh. The liver dry weight was determined by heating a weighed aliquot at 100°C for 48 hours. Pieces from selected organs were fixed in Zenker-formalin solution and in 10% formalin. Paraffin sections were prepared and stained with hematoxylin and eosin. Some were stained with Best's carmine and periodic acid-Schiff stains. Frozen sections of liver were stained with oil red O.

Collection of material

Liver. Pieces of liver from the median and left lateral lobes were removed rapidly, weighed and placed in 30% KOH for glycogen determination. Another weighed piece was homogenized in distilled water and a suitable aliquot was added to an equal volume of 10% trichloroacetic acid (TCA) for protein determination. One large piece of liver was weighed and then frozen at −15°C for subsequent lipid determination.

Gastrocnemius muscle. The right gastrocnemius muscle was removed, weighed and homogenized in distilled water. An aliquot was added to an equal volume of 10% TCA for protein determination.

Pancreas. The whole pancreas was removed rapidly, chilled and weighed. Care was taken to exclude surrounding adipose tissue and lymph nodes. Part of the organ was homogenized in ice-cold 0.02 M phosphate buffer, pH 7.6 and suitable aliquots were added to an equal volume of 10% TCA for protein determination. The remainder was stored at −15°C.

Chemical analyses

Protein. Protein was measured by determination of Kjeldahl nitrogen (Perrin, '53) on the TCA precipitates from suitable aliquots of the liver, pancreas and gastrocnemius muscle. The aliquots were washed in succession with 5% TCA, 95% ethanol, ethanol-ethyl ether mixture (3:1) and ethyl ether before digestion.

Lipid. For total liver lipid, the frozen aliquot of liver was thawed, ground to a dry powder with anhydrous sodium sulfate and extracted with chloroform for 24 hours. After evaporation of the chloroform, the residue was extracted with petroleum ether and the lipid remaining on evaporation of this solvent was weighed.

Aliquots of this lipid residue dissolved in chloroform were used for determinations of cholesterol and phospholipid. Cholesterol was determined by the method of Carr and Drekter ('56), and phospholipid by digesting and measuring phosphorus by the method of Fiske and Subbarow ('25).

Other substances. Liver glycogen was determined on approximately 1 gm aliquots of liver by the method of Seifter et al., ('50). Ribonucleic acid (RNA) determinations on liver were performed by the method of Schmidt and Thannhauser ('46) using measurements both of phosphorus as described by Fiske and Subbarow ('25) and of ultraviolet absorption, as described by Logan et al. ('52). Aliquots of the pancreatic homogenates were analyzed for amylase activity by the method of Smith and Roe ('49). The results are expressed as Smith and Roe units per pancreas.

RESULTS

General condition of animals

In the force-feeding experiments, the control and lysine-devoid groups of rats were normal in general appearance and were indistinguishable from rats fed the stock diet. In contrast, in the animals of the valine-devoid group the hair soon became rough and shaggy, and many of these animals appeared listless and weak. Frequently, rats in this group died after only two or three days of force-feeding and in some experiments up to 50% of the animals died within the first three days. None of the animals force-fed either the control or experimental diets developed diarrhea. The rats in the ad libitum experiments showed no differences between those given the deficient diets and those receiving the complete diet, except that the rats fed the deficient diets weighed less.

Body and organ weights and food consumption

In table 1 are summarized the changes in the weights of the whole body, liver, pancreas, parotid, submaxillary, kidney, gastrocnemius muscle, testis, spleen and thymus in the control and experimental rats force-fed for 5 and 6 days. The control rats gained whereas the experimental rats lost weight, even though the amount of food administered to all animals was the same in each experiment.

The mean wet weight of the liver in animals force-fed the valine-devoid diets, but not those given the lysine-devoid diets, was significantly elevated above that of the controls. The percentage of liver, dry weight, (table 2) was 1.6% less in the valine-devoid group than in the control group.

The weights of the parotid, submaxillary and spleen were significantly less in the rats force-fed the valine devoid diet than in rats on the complete diet. The testis weighed significantly less in rats of the lysine-devoid group than in rats of the control group.

The body and organ weights of animals fed ad libitum for 7 days (table 1) were markedly different from those in the force-fed groups. In contrast to the force-feeding experiments in which all rats received the same amount of ration regardless of the nature of the diet, the animals in the groups fed ad libitum consumed different amounts, depending upon the composition of the diet. Rats in the control groups ate the largest amount of food, 48.3 gm/rat/7 days, and gained weight, whereas those fed the valine-devoid or lysine-devoid diets ate 21.0 gm/rat/7 days and 30.5 gm/rat/7 days respectively and lost weight. In animals of the control groups the liver, parotid, submaxillary, gastrocnemius muscle, kidney, testis, spleen and thymus were heavier than in the animals of either of the experimental groups (table 1). However, the weight of the pancreas was essentially the same in all groups.

Morphologic changes

Seven-day ad libitum experiments. No specific gross or microscopic alterations were found in the animals fed the devoid diets ad libitum for 7 days in comparison with the controls. This is in sharp contrast to the findings in the force-fed groups.

Six-day force-feeding experiments. Liver. The livers of the control animals contained little fat or glycogen when examined with hematoxylin and eosin and with special stains (figs. 1, 3, and 5). The majority of nuclei of the hepatic cells contained several small nucleoli (fig. 7).

TABLE 1

Change in body and organ weights of rats fed control, valine-, or lysine-devoid diets

Group	Rats	Body weight			Liver	Pancreas	Parotid	Sub-maxillary	Gastroc-nemius muscle	Kidney	Testis	Spleen	Thymus
		Onset	Sacrifice	Change									
	no.	gm	gm	gm	gm	mg	mg	mg	mg	mg	mg	mg	mg
Three-day force feeding experiment													
Control	4	79.1	81.9	+2.8	3.01 ±.15[1]	354 ±15[1]	44 ±5[1]	91 ±7[1]	395 ±6[1]	410 ±10[1]	543 ±31[1]	207 ±20[1]	124 ±12[1]
Valine-devoid	7	80.6	76.4	−4.2	3.61 ±.21[2]	389 ±27	43 ±8	68 ±6[2]	378 ±10	417 ±8	482 ±24	207 ±33	153 ±20
Six-day force feeding experiments													
Control	13	72.5	77.7	+5.2	3.14 ±.09	312 ±14	42 ±2	71 ±3	390 ±10	379 ±11	493 ±19	225 ±12	85 ±8
Valine-devoid	11	72.0	69.0	−3.0	3.67 ±.09[2]	340 ±28	28 ±4[2]	54 ±3[2]	340 ±14[2]	367 ±8	442 ±141	130 ±12[2]	78 ±7
Lysine-devoid	14	71.4	71.3	−0.1	3.27 ±.09	355 ±12[2]	34 ±2[2]	66 ±3	335 ±10[2]	356 ±25	352 ±34[2]	187 ±13	63 ±8
Seven-day ad libitum experiments													
Control	8	72.7	88.7	+16.0	4.46 ±.20	331 ±35	46 ±2	89 ±4	448 ±12	448 ±12	615 ±25	405 ±51	258 ±15
Valine-devoid	11	74.0	63.6	−10.4	2.68 ±.16[3]	329 ±12	33 ±2[3]	64 ±3[3]	328 ±12[3]	295 ±16[3]	467 ±18[3]	202 ±10[3]	111 ±6[3]
Lysine-devoid	8	70.7	68.7	−2.0	3.59 ±.13[3]	308 ±15	34 ±2[3]	70 ±3[3]	344 ±9[3]	334 ±9[3]	531 ±43	225 ±9[3]	157 ±20[3]

[1] Mean value ± standard error of the mean.

[2] P between 0.01 and 0.05 (probably significant).

[3] $P < 0.01$ (highly significant).

TABLE 2

Analyses of liver and right gastrocnemius muscle of rats fed control, valine- or lysine-devoid diets[1]

Group	Liver							Right gastrocnemius muscle protein
	Total lipid	Cholesterol	Phospholipid	Neutral fat (by difference)[2]	Glycogen	Protein	Dry weight	
	mg/liver	*mg/liver*	*mg/liver*	*mg/liver*	*mg/liver*	*mg/liver*	%	*mg/muscle*
Three-day force-feeding experiment								
Control	(4) 134 ±19[3]	—	—	—	(4) 20 ±12[3]	(4) 567 ±14[3]	(4) 30.2 ±0.4[3]	(4) 70.5 ±2.6[3]
Valine-devoid	(7) 162 ±4	—	—	—	(7) 55 ±20	(7) 594 ±27	(4) 28.8 ±0.6	(7) 65.9 ±1.7
Six-day force-feeding experiments								
Control	(13) 145 ±9	(11) 10.7 ±0.5	(11) 64.0 ±2.7	(11) 64.4 ±6.0	(13) 77 ±17	(7) 525 ±14	(5) 30.3 ±0.5	(5) 70.9 ±2.9
Valine-devoid	(7) 221 ±22[4]	(7) 12.5 ±1.1	(7) 74.5 ±2.4[5]	(7) 133.3 ±16.2[4]	(5) 161 ±29[5]	(5) 493 ±28	(5) 28.7 ±0.2[5]	(5) 57.1 ±3.3[5]
Lysine-devoid	(14) 172 ±14	(13) 11.6 ±0.9	(13) 59.7 ±4.8	(13) 105.5 ±13.4[5]	(11) 127 ±26	(6) 538 ±15	(3) 29.2 ±0.7	(5) 64.8 ±3.9
Seven-day ad libitum experiments								
Control	(7) 201 ±21	—	—	—	(8) 371 ±48	(8) 580 ±22	(8) 31.1 ±0.5	(7) 87.3 ±3.0
Valine-devoid	(11) 84 ±4[4]	—	—	—	(8) 201 ±32[5]	(11) 407 ±15[4]	(7) 30.2 ±0.6	(11) 60.6 ±2.4[4]
Lysine-devoid	(8) 153 ±18	—	—	—	(8) 321 ±28	(8) 425 ±17[4]	(4) 30.1 ±0.1	(8) 61.1 ±1.6[4]

[1] Numbers in parentheses indicate number of animals in group.

[2] Neural fat = total liver lipid − (cholesterol plus phospholipid). See text for explanation.

[3] Mean value ± standard error of the mean.

[4] $P < 0.01$ (highly significant).

[5] P between 0.01 and 0.05 (probably significant).

In the rats fed the valine-devoid diet, the liver was large and had a glistening yellowish-brown appearance. Histologically, the hepatic cells contained vacuolated cytoplasm especially in the periportal areas (fig. 2). With special stains, the liver cells showed considerable amounts of both lipid and glycogen (figs. 4 and 6). The lipid was confined to the hepatic cells about the portal triads, whereas the glycogen was diffusely distributed. The hepatic cell nuclei in most cases contained one large nucleolus instead of several small ones (fig. 8). The livers of the animals force-fed the lysine-devoid diet were similar to those given the valine-devoid diet but the changes were less marked.

Pancreas. The pancreas was normal in the control animals (fig. 9). In rats of the valine-devoid and lysine-devoid groups the acinar cells of the pancreas showed moderate loss of cytoplasm and of zymogen granules. The nuclei were somewhat disorderly in arrangement and were crowded together (fig. 10). The islets were normal. These changes were found in the majority of experimental animals but not in all of them.

Parotid gland. In the animals of both the valine- and the lysine-devoid groups, the parotid glands of most animals were smaller than normal. On microscopic examination, a striking loss of cytoplasm was observed in the glands of the experimental animals (fig. 12) in comparison with the glands of the control animals (fig. 11).

Stomach. On microscopic examination the forestomach and glandular part of the stomach of the control and lysine-devoid groups were normal. Abundant mucinous material was demonstrable in the glands of the upper mucosa with the periodic acid-Schiff stain (fig. 13). In contrast, the gastric glands in rats force-fed the valine-devoid diet showed almost complete absence of mucin (fig. 14). No changes were present in the forestomach.

Thymus gland and spleen. The thymus gland was normal in the control animals (fig. 15). In contrast the thymus glands of the animals force-fed the valine- and lysine-devoid diets were smaller and histologically showed diminution of lymphocytes in the cortex and loss of distinction between cortex and medulla (fig. 16). These findings are usual in atrophy of the thymus gland from a variety of causes. Atrophic changes were also found in the spleen of the two experimental groups and consisted in a reduction in lymphocytes and prominence of the connective tissue. The spleen in the control group was normal.

The following organs showed no gross or microscopic changes: heart, kidney, gastrocnemius muscle, small intestine, large intestine, submaxillary gland, adrenal and lung.

Autopsies of animals in the valine-devoid group which died after two or three days of force-feeding showed marked distention of the intestinal tract but no other findings.

Three-day force-feeding experiments. In one force-feeding experiment rats were killed after being given the control or valine-devoid diet for three days. Sections of liver stained with oil red O revealed a moderate increase of lipid in hepatic cells in the periportal areas in animals fed the devoid diet. The pancreas and parotid gland showed early atrophic changes in the experimental animals. Other organs were essentially normal.

Biochemical changes

Rats force-fed valine- or lysine-devoid diets for 6 days. Liver. The liver weight changes are summarized in table 1. A summary of the liver lipid, glycogen and protein content of the animals in the valine-devoid, lysine-devoid and control groups is shown in table 2. The total liver lipid content was significantly greater in the valine-devoid group than in the control group. Analysis of the lipid revealed significant changes in the liver phospholipid and neutral fat. The liver cholesterol was slightly but insignificantly increased. The total liver lipid content was slightly greater in the lysine-devoid group than in the control group and was largely due to the increase in neutral fat. The neutral fat values were calculated by subtracting the cholesterol and phospholipid values from the total liver lipid and therefore were obtained only in those animals in which cholesterol and phospholipid were determined.

Liver glycogen was determined as a check against the histologic findings of

increased liver glycogen in the animals on the valine- and lysine-devoid diets. There was a probable significant increase in liver glycogen content in the animals of the valine-devoid group and only a moderate increase in animals of the lysine-devoid group over that in the control animals.

The liver protein analysis was interesting in that the protein content in the animals of the valine- and lysine-devoid groups was essentially the same as that in the animals of the control group.

In one experiment liver ribonucleic acid (RNA) was determined. The liver RNA content in the rats on the complete diet and in the rats on the valine-devoid diet was 2.41 and 2.60, respectively. The results are expressed as milligrams of RNA-P per liver.

Skeletal muscle. The results obtained by analyzing the right gastrocnemius muscle of rats in three-day and 6-day experiments are summarized in table 2. By three days there is a slight decrease whereas at 6 days there is a significant decrease in muscle weight and protein content in the valine-devoid group in comparison with the control group. At 6 days there is likewise a significant decrease in muscle weight but not of protein in the lysine-devoid group in comparison with the control group.

Pancreas. In one experiment, analyses of pancreatic protein and amylase were performed. The control rats had an average pancreatic protein of 49.3 mg, and the valine-devoid, an average value of 47.8 mg. The amylase activity of the pancreas from the control animals was 6,680, and from the animals fed the valine-devoid diet it was 2,340. The amylase units are expressed as Smith and Roe units per pancreas.

Rats fed valine- or lysine-devoid diets ad libitum for 7 days. The changes in body and organ weights in animals fed the valine-devoid, lysine-devoid or control diets ad libitum are described in table 1. In these ad libitum experiments the experimental animals showed a diminution in all of these values except for pancreas weight.

In table 2 are summarized the values of liver lipid, glycogen and protein content, and right gastrocnemius muscle protein content in the experimental and control groups. The liver lipid, glycogen and protein content was markedly decreased in animals of the valine-devoid group and only moderately decreased in the lysine-devoid group as compared with the same components in animals of the control group. The wet weight and protein content of the gastrocnemius muscle was significantly less in the experimental animals than in the control animals.

In one experiment, liver RNA analyses were performed. The liver of animals fed the complete diet contained an average of 2.48 mg of RNA-P whereas the liver of the animals on the valine-devoid diet contained an average of 1.60 mg of RNA-P.

Rats force-fed the valine-devoid diet for three days. Since many striking changes were found in the valine-devoid experiments of 6 days' duration and since many of the experimental animals died in the first few days an attempt was made to learn how early many of these changes might occur. Therefore a group of animals was killed at the end of three days. The findings are summarized in tables 1 and 2. There was probably a significant increase in the wet weight of the liver and a suggestive increase in liver lipid and glycogen in animals given the valine-devoid diet as compared with those given the control diet. These three-day results indicate that many of the changes found at 6 days had already begun within three days.

DISCUSSION

The results of this study show that pathologic changes in the liver, pancreas, parotid, stomach, thymus and spleen can be induced rapidly in young rats when the animals are force-fed a purified diet devoid of valine. The pathologic changes in animals force-fed a lysine-devoid diet were similar but less striking. On the other hand, when animals were fed the identical valine- or lysine-devoid diets ad libitum, few if any pathologic changes were found. In addition, marked differences were found between the chemical analyses of certain organs when the animals were force-fed and when they were fed ad libitum the same diets.

The animals that were force-fed the valine- or lysine-devoid diets lost body weight

but showed an increase in liver weight, lipid and glycogen with no change in liver protein in comparison with the animals force-fed the complete diet. In contrast, the rats fed the same devoid diets ad libitum showed a decrease in all liver values when compared with control animals that ate the complete diet ad libitum. These differences appear to be related to the quantity of the deficient diet consumed. The force-fed animals received the same amount of the different diets in all groups while under ad libitum feedings the animals fed the devoid diets consumed much less than the animals receiving the complete diet.

Results obtained from our own series of experiments and from experiments by others with rats force-fed single essential amino acid deficient diets demonstrate certain similar pathologic and chemical findings among many amino acid deficiencies. On pathologic examination certain organs such as the liver, pancreas and salivary glands have consistently shown the same abnormalities. The most constant and striking of all the pathologic findings has been the fatty change of the liver. The fatty liver has a distinct periportal distribution of lipid and has been described in the liver of rats force-fed diets deficient in tryptophan[8] (Adamstone and Spector, '50; Van Pilsum et al., '57), methionine (Sidransky and Farber, '58a, c), phenylalanine (Samuels et al., '51; Van Pilsum et al., '57), threonine (Sidransky and Farber, '58a, c), and histidine (Sidransky and Farber, '58a). The same fatty alteration has now been found with deficiencies of valine and lysine. In addition to the increase in liver lipid, an increase in liver glycogen has frequently been observed. Also it is noteworthy that in many short-term experiments no loss of protein in the liver but a marked loss of muscle protein was observed in rats fed the deficient diets. These similar and frequent findings observed in rats force-fed diets deficient in different essential amino acids suggest that deficiencies of many essential amino acids produce certain changes in common. These alterations probably reflect the effect of overall protein deficiency rather than of a specific amino acid deficiency.

Certain data demonstrate specific differences among the amino acid deficiencies. Some of these differences are in rate of growth (Sugimura et al., '59), in survival (Sidransky and Farber, '58a; Sugimura et al., '59), in certain biochemical alterations (Denton et al., '50; Bothwell and Williams, '51, '52; Sidransky and Farber, '58b; Sugimura et al., '59), and in some histopathologic changes (Adamstone and Spector, '50; Sidransky and Farber, '58a). These dissimilar findings suggest that certain variations are probably related to deficiencies of specific amino acids.

In our previous communications of this series (Sidransky and Farber, '58a, b) we discussed in detail many of our concepts concerning the major pathologic and chemical findings in rats force-fed diets devoid of certain single essential amino acids, threonine, methionine and histidine. Our present results with young rats force-fed diets devoid of valine or lysine add further support to these views. It appears to be more than coincidence that the findings in young rats in our force-feeding experiments closely resemble the clinical and pathologic findings in human cases of kwashiorkor. The force-feeding of nutritionally-deficient or imbalanced diets may be a helpful tool in gaining some understanding of such complex human disorders as kwashiorkor.

SUMMARY

Rats force-fed a purified diet devoid in valine were found to develop a periportal fatty liver, excess hepatic glycogen, and atrophy of the pancreas, parotid, thymus and spleen within 6 days after beginning the diets. Rats force-fed the lysine-devoid diet showed similar but less striking changes. It was found that the animals force-fed the valine- or lysine-devoid diet showed no change of protein content in the liver but a marked decrease of protein content in the right gastrocnemius muscle in comparison with animals given the complete diet. Animals fed the same deficient diets ad libitum, in contrast with those force-fed, consumed less food, showed loss of muscle and liver protein with a decrease

[8] See footnote 2, p. 463.

of liver lipid and glycogen, and showed no specific pathologic changes. The differences in the results between the force-feeding and the ad libitum feeding regimens are explained in terms of differences in the quantity of the deficient diets consumed. The pathologic lesions found in the rats force-fed valine- or lysine-devoid diets resembled those described to be characteristic of kwashiorkor.

ACKNOWLEDGMENTS

The authors are greatly indebted to Mrs. Shirley Clark for technical assistance in the study.

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PLATE 1

EXPLANATION OF FIGURES

1 Liver of control rat. Hematoxylin and eosin. × 260.

2 Liver of rat force-fed the valine-devoid diet. Lipid vacuolation is prominent in hepatic cells in periportal area (left). Only a few vacuoles are present in central area (right). Compare with figure 1. Hematoxylin and eosin. × 260.

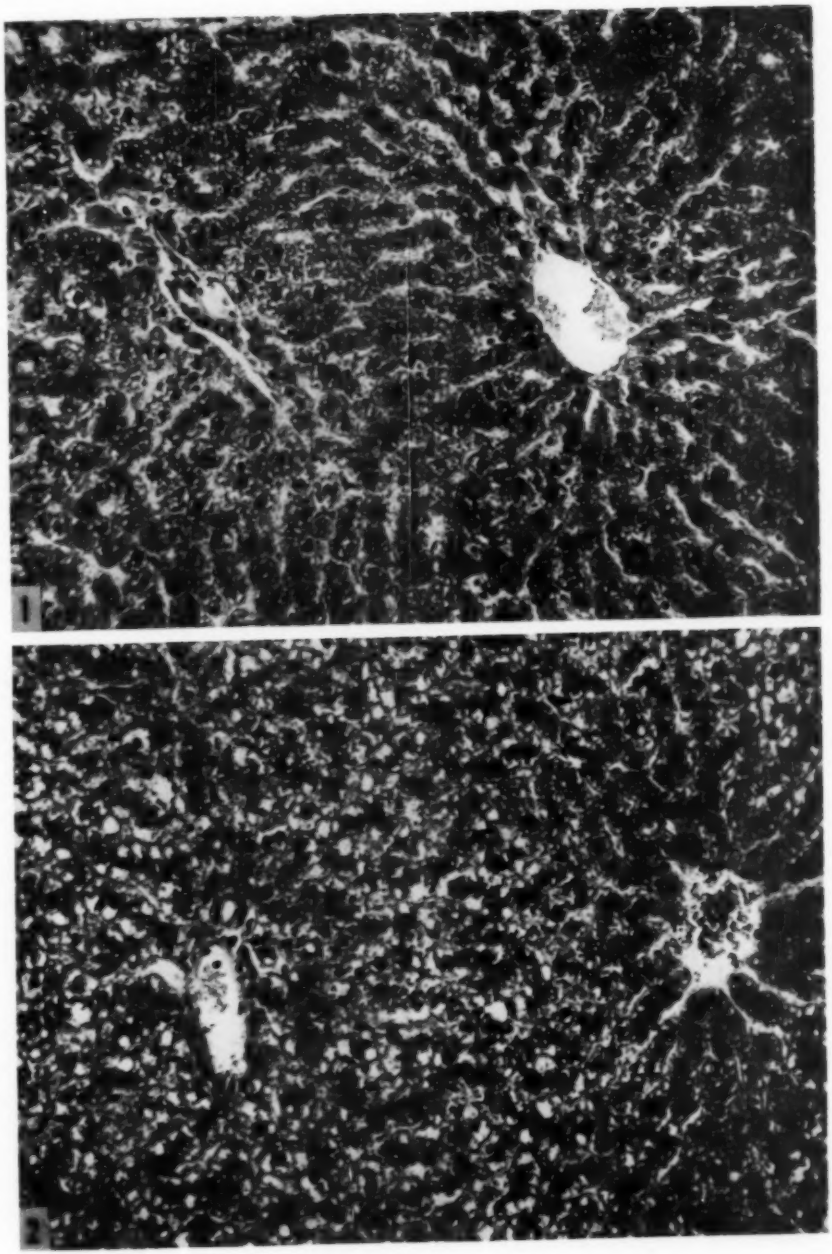
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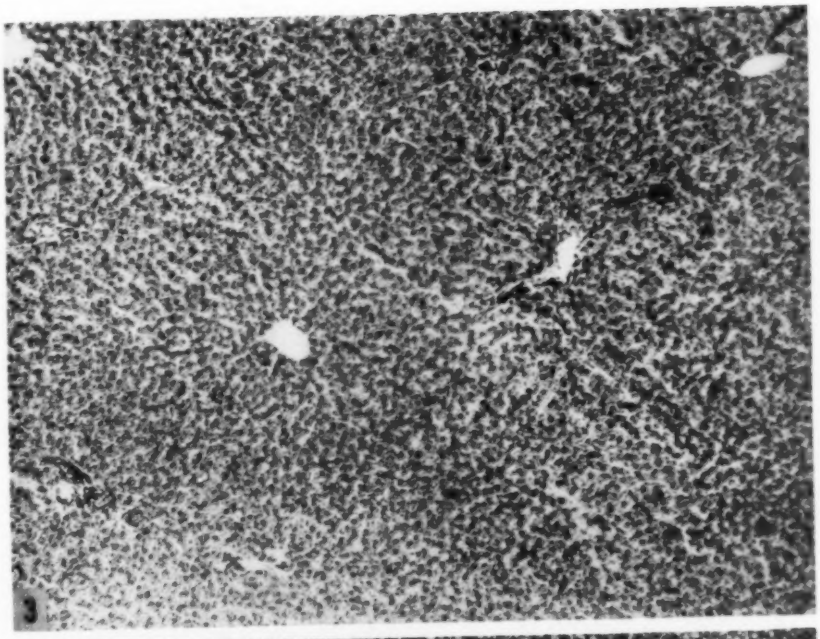
PLATE 2

EXPLANATION OF FIGURES

3 Liver of control rat. Oil red O stain. × 85.

4 Liver of rat force-fed the valine-devoid diet. Lipid is prominent in hepatic cells in periportal areas. Little lipid is present in cells about the central areas. Compare with figure 3. Oil red O stain. × 85.



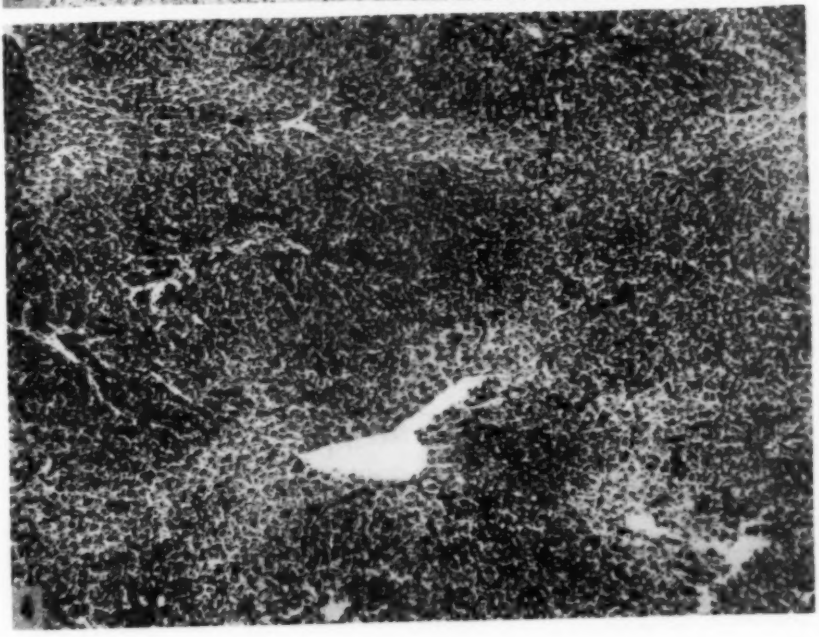


PLATE 3

EXPLANATION OF FIGURES

5 Liver of control rat. Best's carmine stain. × 85.

6 Liver of rat force-fed the valine-devoid diet. A large amount of glycogen is present throughout. Compare with figure 5. Best's carmine stain. × 85.

7 Liver of control rat. Oil-immersion view of hepatic cells showing nuclei containing many small nucleoli. Hematoxylin and eosin. × 1700.

8 Liver of rat force-fed the valine-devoid diet. Oil-immersion view of hepatic cells, showing nuclei containing single large nucleolus. Hematoxylin and eosin. × 1700.

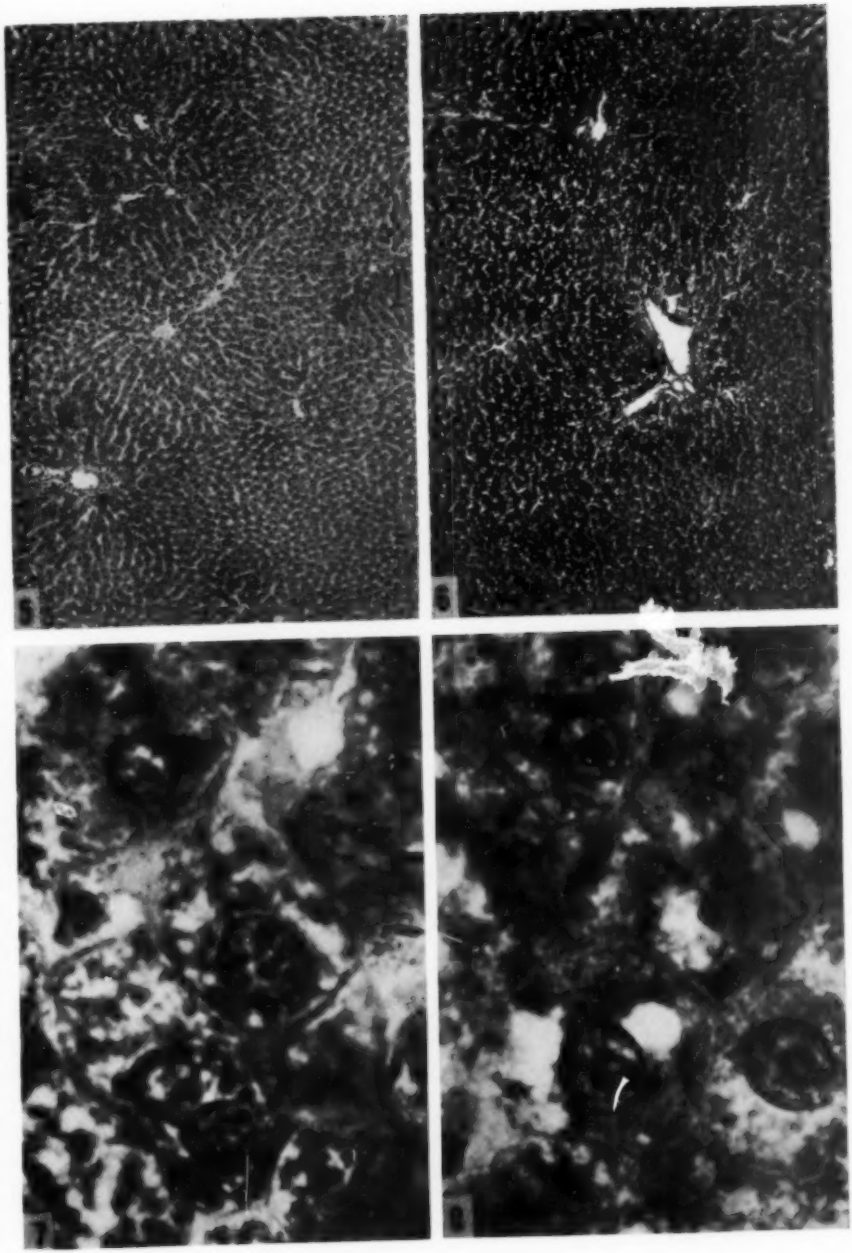
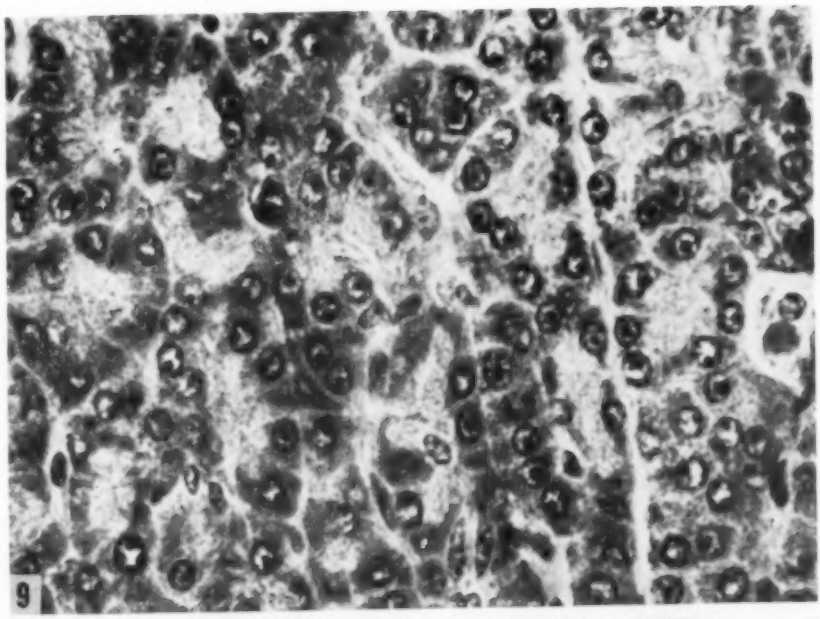


PLATE 4

EXPLANATION OF FIGURES

9 Pancreas of control rat. Hematoxylin and eosin. × 830.

10 Pancreas of rat force-fed the valine-devoid diet. Note reduction of cytoplasm and zymogen granules of acinar cells. Compare with figure 9. Hematoxylin and eosin. × 830.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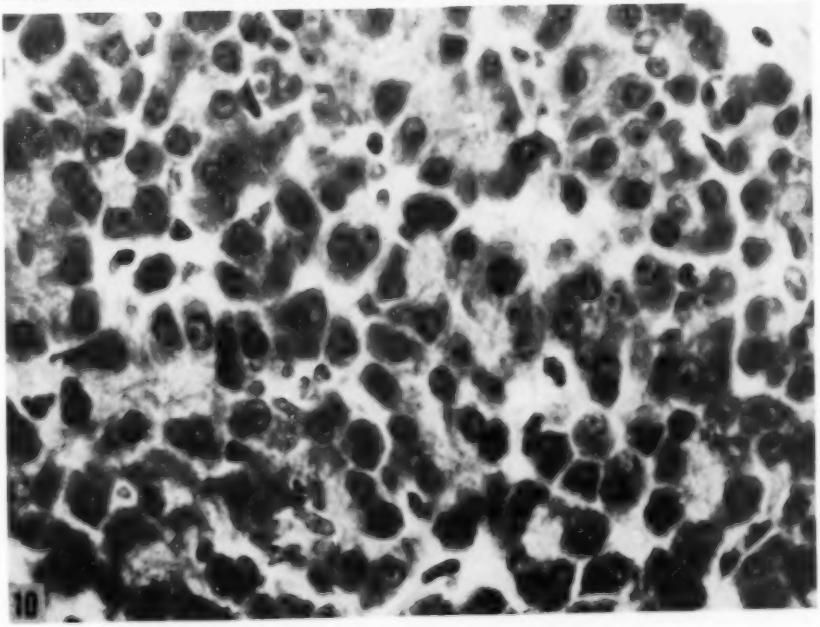
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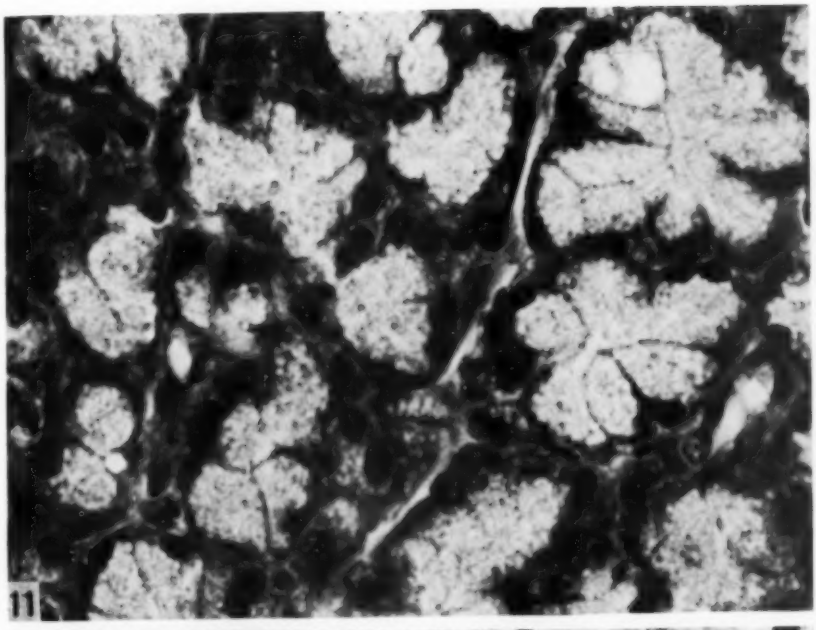
PLATE 5

EXPLANATION OF FIGURES

11 Parotid gland of control rat. Hematoxylin and eosin. × 830.

12 Parotid gland of rat force-fed the valine-devoid diet. Note loss of cytoplasm of the acinar cells. Compare with figure 11. Hematoxylin and eosin. × 830.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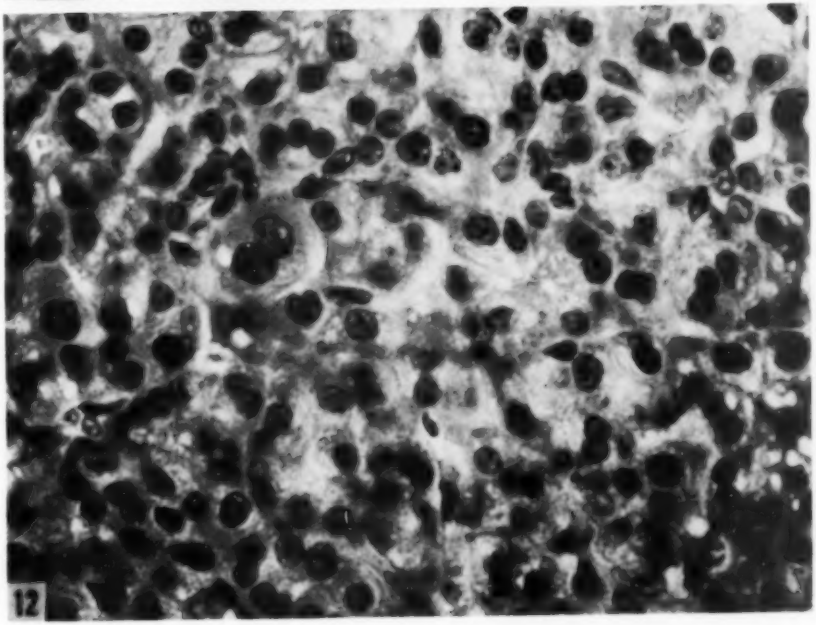
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PLATE 6

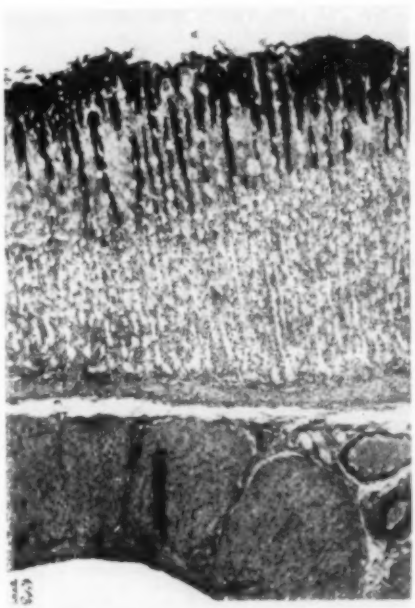
EXPLANATION OF FIGURES

13 Stomach of control rat. Note the abundant mucin in the upper glandular cell. Periodic acid-Schiff stain. × 90.

14 Stomach of rat force-fed the valine-devoid diet. Note the small amount of mucin. Compare with figure 13. Periodic acid-Schiff. × 90.

15 Thymus of control rat. Hematoxylin and eosin. × 90.

16 Thymus of rat force-fed the valine-devoid diet. Note atrophic changes with loss of differentiation of the cortical and medullary areas and diminution of lymphocytes. Compare with figure 15. Hematoxylin and eosin. × 90.



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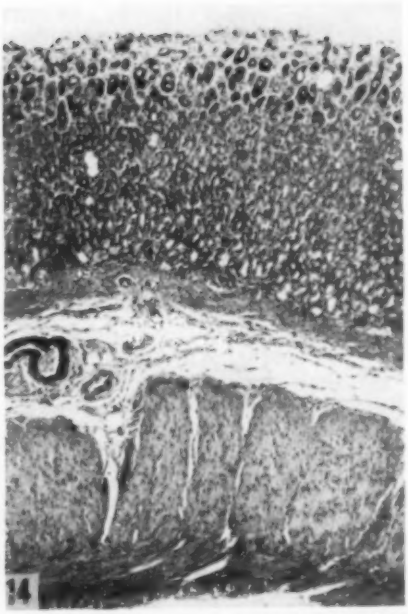

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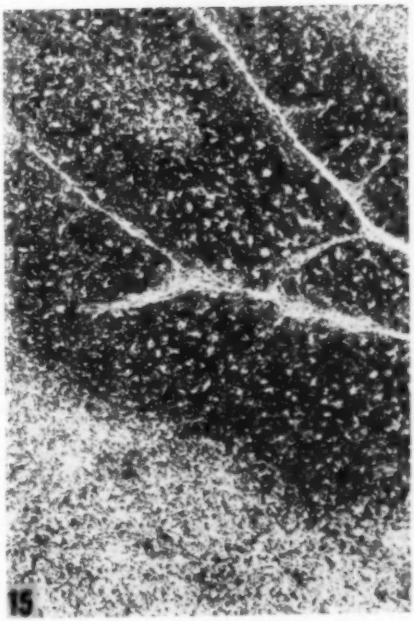

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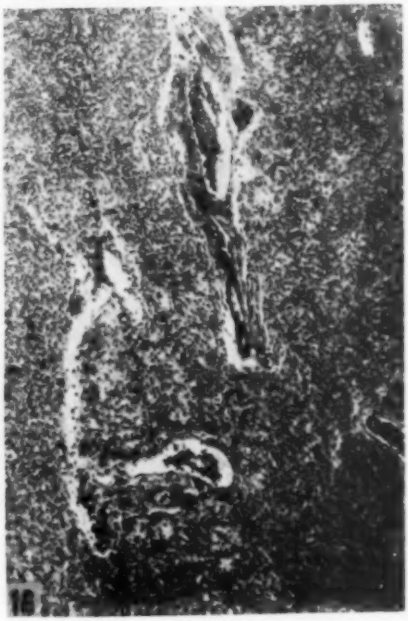

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Beneficial Effects of Alfalfa Meal and Other Bulk-Containing or Bulk-Forming Materials on the Toxicity of Non-Ionic Surface-Active Agents in the Rat[1]

BENJAMIN H. ERSHOFF
Department of Biochemistry and Nutrition, University of Southern California, Los Angeles

Although a number of investigators have reported that non-ionic surface-active agents have toxic effects when fed at levels of 5 to 25% of the ration (Schweigert et al., '50; Harris et al., '51a,b; Chow et al., '53; Poling et al., '56; Brush et al., '57), others have reported that such materials may be fed for prolonged periods with little if any deleterious effect (Krehl et al., '55; Graham et al., '54; Graham and Grice, '55; Oser and Oser, '56a,b, '57a,b). There is evidence that the diverse results indicated above were due, at least in part, to differences in the diets fed. Thus, Chow et al. ('53) observed that whereas a supplement of 5% polyoxyethylene (20) sorbitan monostearate (Tween 60) resulted in growth retardation and diarrhea when fed to weanling rats in conjunction with a highly purified (casein and sucrose-containing) ration, no deleterious effects were observed following the administration of this surfactant even at a 15% level when fed with a diet containing soybean meal. It was suggested by these workers that the increased toxicity of the Tween 60 when fed with the purified diet was due to the lack of sufficient residues in the ration used to absorb the surface-active agent, which was irritating to the intestinal tract by virtue of its physical properties. As evidence for this hypothesis they cite their finding that supplementing the purified diet with bulk-forming inert substances such as celluflour,[2] Celite[3] or agar prevented the occurrence of diarrhea (Chow et al., '53). Data indicating that bulk-containing or bulk-forming materials were effective in counteracting the toxic effects of surfactants when fed with a purified low-fiber diet have also been reported for the mouse (Ershoff and Hernandez, '59). Desiccated alfalfa and other succulent plants, carrageenin, sodium alginate and agar were particularly effective in this regard (Ershoff and Hernandez, '59). In the present communication further data are presented on the comparative effects of alfalfa and other bulk-containing or bulk-forming materials on symptoms of toxicity associated with the feeding of high levels of surface-active agents in the rat.

PROCEDURE AND RESULTS

The basal ration used in these experiments consisted of sucrose, 66%; casein,[4] 24%; salt mixture,[5] 5%; and cottonseed oil, 5%. To each kilogram of the above diet were added the following vitamins: thiamine·HCl, 20 mg; riboflavin, 20 mg; pyridoxine·HCl, 20 mg; Ca pantothenate, 60 mg; nicotinic acid, 100 mg; ascorbic acid, 200 mg; biotin, 4 mg; folic acid, 10 mg; *p*-aminobenzoic acid, 400 mg; inositol, 800 mg; vitamin B_{12}, 150 μg; 2-methyl-1,4-naphthoquinone, 5 mg; choline chloride, 2 gm; vitamin A, 5000 U.S.P. units; vitamin D_2, 500 U.S.P. units; and

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[2] Cellu Flour, Chicago Dietetic Supply House.

[3] Diatomaceous silica filter aid, Johns-Manville, New York.

[4] Vitamin-free Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

[5] Salt Mixture Wesson Modification (Osborne-Mendel), General Biochemicals, Inc., Chagrin Falls, Ohio.

TABLE 1

Growth and survival of immature rats fed massive doses of Myrj 45, Myrj 52, Tween 20, Tween 60 and Span 20 with and without alfalfa meal (6 animals per group)[1]

Supplements fed with basal ration	Average gain in body weight after following days of feeding			Percentage of survival[2]
	7	14	21	
	gm	*gm*	*gm*	
None	30.4	61.9	85.5	100
15% Myrj 45	23.8	57.0	71.6	100
20% Myrj 45	20.8	50.2	74.2	100
15% Myrj 45 + 20% alfalfa meal	24.2	58.0	80.5	100
20% Mryj 45 + 20% alfalfa meal	31.5	69.0	94.3	100
15% Myrj 52	4.6	14.5(3)[3]	28.1(3)	50
20% Myrj 52	1.6(5)	30.8(2)	45.3(2)	33.3
15% Myrj 52 + 20% alfalfa meal	30.7	61.7	87.0	100
20% Myrj 52 + 20% alfalfa meal	31.3	66.7	93.8	100
15% Tween 20	3.5(2)	—	—	0
20% Tween 20	1.3(4)	—	—	0
15% Tween 20 + 20% alfalfa meal	34.3	69.2	97.5	100
20% Tween 20 + 20% alfalfa meal	31.1	63.1	92.8	100
15% Tween 60	5.8(4)	—	—	0
20% Tween 60	—	—	—	0
15% Tween 60 + 20% alfalfa meal	36.7	73.0	96.7	100
20% Tween 60 + 20% alfalfa meal	32.2	70.8	100.7	100
15% Span 20	18.0	40.4	58.5	100
20% Span 20	1.5(2)	8.5(2)	27.0(2)	33.3
15% Span 20 + 20% alfalfa meal	17.0	35.5	54.0	100
20% Span 20 + 20% alfalfa meal	3.3(4)	14.8(4)	26.8(4)	66.7

[1] The average initial body weight of rats in the various groups ranged between 47.4 and 48.2 gm.

[2] Experimental period, 21 days.

[3] The values in parentheses indicate the number of animals alive at the time and on which averages are based. These values are given only for groups where the number per group differs from the number started.

α-tocopheryl acetate, 100 mg. The vitamins were added in place of an equal amount of sucrose. Tests were conducted with the following surfactant agents: polyoxyethylene stearate (Myrj 45); polyoxyethylene stearate (Myrj 52); polyoxyethylene sorbitan monolaurate (Tween 20); polyoxyethylene sorbitan monostearate (Tween 60); and sorbitan monolaurate (Span 20).[6] The above surfactant agents as well as the various test supplements were incorporated in the basal ration in the amounts listed in tables 1 to 3, replacing equal amounts of sucrose.

Female rats of the Holtzman strain were selected, 21 to 24 days old, having a body weight between 40 and 54 gm. The rats were housed in metal cages with raised screen bottoms (two animals per cage) and provided with food and water ad libitum. The animals were fed daily and all food not consumed 24 hours after feeding was discarded. Feeding was continued for 21 days or until death, whichever occurred earlier.

Experiment 1. Growth and survival of immature rats fed massive doses of Myrj 45, Myrj 52, Tween 20, Tween 60 and Span 20 with and without alfalfa meal.

[6] These surfactants were obtained from the Chemicals Division, Atlas Powder Company, Wilmington, Delaware, and had HLB (hydrophile-lipophile balance) values of 11.1, 16.9, 16.7, 14.9 and 8.6, respectively. The HLB system as developed by the Atlas Powder Co. is based on the premise that all surfactants have both hydrophilic and lipophilic groups in one molecule. The balance between these groups is an indication of whether the surfactant will form an oil-in-water or a water-in-oil emulsion. The lower the HLB value, the more lipophilic is the material. Conversely, the higher the HLB value, the more hydrophilic is the material. Those in the HLB range of 10 to 11 are intermediate. In general, materials of low HLB value tend to be oil-soluble, and those of high HLB tend to be water-soluble. Although both Myrj 45 and Myrj 52 were similar chemically (i.e., they were both polyoxyethylene stearates), they differed significantly in respect to HLB values.

A significant difference in response was observed between rats fed the various surfactant agents both with and without alfalfa meal supplement. Myrj 52, Tween 20 and Tween 60, when fed at a 15 or 20% level in the basal ration, resulted in a highly significant retardation in growth and a decreased incidence of survival. The growth retardation was evident during the first week of feeding and was accompanied by diarrhea, an unthrifty appearance and varying degrees of alopecia. These effects were counteracted completely by the concurrent administration of alfalfa meal at a 20% level in the diet. Rats fed the above surfactants with alfalfa meal appeared normal grossly in all respects and were indistinguishable in appearance from those fed the basal ration without surfactants. Span 20, when fed at a 15 or 20% level in the basal ration, also resulted in growth retardation and other symptoms of toxicity comparable to those indicated above. Alfalfa meal, however, did not counteract the toxic effects obtained with Span 20, in contrast to its protective effect in rations containing Myrj 52, Tween 20 and Tween 60.[7] Myrj 45, when fed at a 15 or 20% level in the basal ration, resulted in only a slight retardation in growth and had no deleterious effects on either gross appearance or survival during the 21-day experimental period. Results are summarized in table 1.

Experiment 2. Comparative effects of alfalfa meal, alfalfa fractions and supplements of the known nutrients on the weight increment and percentage of survival of immature rats fed massive doses of Myrj 52, Tween 20 and Tween 60. In agreement with experiment 1, Myrj 52, Tween 20 and Tween 60, when fed at a 15% level in the basal ration, resulted in a highly significant retardation in growth and a decreased incidence of survival. In further agreement with experiment 1, these effects were completely counteracted by the concurrent administration of alfalfa meal at a 20% level in the diet. Alfalfa meal, when fed at a 10% level in the diet, had similar activity. The protective factor or factors in alfalfa was retained in the alfalfa-residue fraction (the water-washed pulp remaining after the extraction of the juice). Dried alfalfa juice when fed at a 5% level in the diet (corresponding to the amount provided by a 20% alfalfa meal supplement) had little if any activity. A supplement of the known nutrients which increased the casein content of the diet by 10% of the ration, the cottonseed oil content by 5% of the ration, the salt mixture content by 2.5% of the ration, as well as doubling the vitamin content of the diet was without significant protective effect. Both growth and survival were significantly improved when cellulose[8] was incorporated at a 10% level in the ration, a finding in agreement with the previously reported data of Chow et al. ('53). The growth-promoting effect of cellulose was less, however, in the case of each of the surfactants tested than that obtained with a comparable amount of alfalfa meal. Results are summarized in table 2.

Experiment 3. Comparative effects of alfalfa meal and other bulk-containing or bulk-forming materials on the weight increment of immature rats fed toxic doses of Tween 60. In agreement with experiments 1 and 2, growth was significantly retarded in immature rats fed the basal ration supplemented with 15% of Tween 60. In addition, rats fed the latter diet exhibited diarrhea, an unthrifty appearance and various degrees of alopecia similar to that observed in the previous experiments, although mortality was less in the present series.[9] The deleterious effects of Tween 60 administration were counteracted completely by the concurrent feeding of alfalfa meal at a 10% level in the diet (fig. 1). Alfalfa meal at a 5 or 2.5% level in the diet was also active although less than at the 10% level of feeding. In agreement with earlier findings, cellulose when added to the diets in the form of solka floc, cel-

[7] Findings indicate that alfalfa meal had significant activity in counteracting the toxic effects obtained on feeding surfactants with high HLB values (i.e., Myrj 52, Tween 20 and Tween 60) but was without significant effect on the toxicity obtained with a surfactant of low HLB value (i.e., Span 20).

[8] Solka Floc BW 200, Brown Company, Boston.

[9] The Tween 60 used in this experiment was of a different lot number than that used in the earlier experiments.

TABLE 2

Comparative effects of alfalfa meal, alfalfa fractions and supplements of the known nutrients on the weight increment and percentage of survival of immature rats fed massive doses of Myrj 52, Tween 20 and Tween 60 (6 animals per group)[1,2]

Supplements fed with basal ration	Average gain in body weight after following days of feeding			Percentage of survival[3]
	7	14	21	
	gm	*gm*	*gm*	
None	30.6	62.2	84.6	100
15% Myrj 52	6.8	27.4	55.5(4)[4]	66.7
15% Myrj 52 + following supplements:				
20% alfalfa meal	32.6	72.8	103.0	100
10% alfalfa meal	32.0	65.2	103.0	100
15% alfalfa residue	29.8	63.2	97.0	100
5% alfalfa juice	6.0	25.7(3)	53.7(3)	50
Known nutrients[5]	8.1	25.4(5)	66.7(3)	50
10% cellulose[6]	23.8	58.0	81.8	100
15% Tween 20	6.0	14.7(3)	29.9(2)	33.3
15% Tween 20 + following supplements:				
20% alfalfa meal	30.6	71.2	104.6	100
10% alfalfa meal	28.2	66.2	98.2	100
15% alfalfa residue	27.6	57.0	91.0	100
5% alfalfa juice	3.4	9.8(1)	32.8(1)	16.7
Known nutrients[5]	3.3(5)	11.7(2)	—	0
10% cellulose[6]	8.8	31.7	48.2	100
15% Tween 60	5.4	13.3(2)	21.3(2)	33.3
15% Tween 60 + following supplements:				
20% alfalfa meal	30.1	69.8	99.5	100
10% alfalfa meal	30.8	71.5	99.1	100
15% alfalfa residue	28.2	58.8	91.0	100
5% alfalfa juice	8.2	30.8	55.8	100
Known nutrients	5.2(3)	15.2(3)	31.7(2)	33.3
10% cellulose	13.0	44.2	65.2	100

[1] The average initial body weight of rats in the various groups ranged between 44.8 and 46.4 gm.

[2] The alfalfa samples were kindly provided by the Research and Development Division of Nutrilite Products, Inc., Buena Park, California.

[3] Experimental period, 21 days.

[4] The values in parentheses indicate the number of animals alive at the time and on which averages are based. These values are given only for groups where the number per group differs from the number started.

[5] The following nutrients were added per kilogram of ration in place of an equal amount of sucrose: Vitamin-free Test Casein, 100 gm; cottonseed oil, 50 gm; salt mixture (Wesson modification), 25 gm; thiamine·HCl, 20 mg; riboflavin, 20 mg; pyridoxine·HCl, 20 mg; Ca pantothenate, 60 mg; nicotinic acid, 100 mg; ascorbic acid, 200 mg; biotin, 4 mg; folic acid, 10 mg; *p*-aminobenzoic acid, 400 mg; inositol, 800 mg; vitamin B_{12}, 150 μg; 2-methyl-1,4-naphthoquinone, 5 mg; choline chloride, 2 gm; vitamin A, 5000 U.S.P. units; vitamin D_2, 500 U.S.P. units; and α-tocopheryl acetate, 100 mg.

[6] Solka Floc BW 200, Brown Co., Boston.

lophane spangles[9] or alphacel[10] had significant activity in counteracting the toxic effects of Tween 60 administration in the immature rat. The protective effect of cellulose was proportional to the level fed; but at each level of feeding between 2.5 and 10% of the diet its protective effect was less than that of a comparable amount of alfalfa meal.

In addition to alfalfa meal, rye grass, fescue grass, oat grass, orchard grass and wheat grass when fed at a 10% level in the diet also had significant activity which was at least equal to that of alfalfa meal

[10] Cellophane Spangles, Rayon Processing Company, Pawtucket, Rhode Island.

[11] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

in counteracting Tween 60 toxicity in the imature rat. Carrageenin, a water-soluble hydrocolloid extracted from Irish Moss, and sodium alginate were also active in this regard. Some activity was exhibited also by pectin and yeast although less than with the other supplements indicated above. Celite and desiccated liver, when fed at a 10% level in the diet had little, if any, protective effect.

Fig. 1 The rat on the right received the basal ration plus 15% Tween 60; the rat on the left received a similar diet supplemented with 10% alfalfa meal. The photograph was taken after 15 days feeding.

TABLE 3

Comparative effects of alfalfa meal and other bulk-containing or bulk-forming materials on the weight increment of immature rats fed toxic doses of Tween 60[1,2]

Supplements fed with basal ration	Number of animals	Average gain in body weight after following days of feeding			Percentage of survival[3]
		7	14	21	
		gm	*gm*	*gm*	
None	12	30.4	63.1	92.0	100
15% Tween 60	18	6.5	21.1(14)[4]	39.3(12)	66.7
15% Tween 60 plus following supplements:					
1% cellulose[5]	12	7.9	28.2	60.4	100
2.5% cellulose[5]	12	11.0	35.0	66.7	100
5% cellulose[5]	12	13.7	40.2	73.9	100
10% cellulose[5]	12	23.5	59.2	86.3	100
2.5% alfalfa meal	12	14.8	41.5	76.8	100
5% alfalfa meal	12	18.9	55.5	88.1	100
10% alfalfa meal	12	27.0	66.5	95.8	100
10% rye grass	6	27.0	67.4	95.2	100
10% fescue grass	6	29.3	70.7	102.2	100
10% oat grass	6	29.2	69.4	103.2	100
10% orchard grass	6	33.0	74.2	105.5	100
10% wheat grass	6	35.3	74.9	105.9	100
10% carrageenin	6	34.2	74.2	108.4	100
10% sodium alginate	6	27.0	64.2	92.5	100
10% cellophane spangles	6	24.0	55.0	86.5	100
10% Alphacel	6	21.8	52.5	72.5	100
10% pectin N.F.	12	23.1	46.9	70.3	100
5% pectin N.F.	6	11.7	37.0	65.0	100
10% Celite	6	9.6	27.2	42.2	100
5% Celite	6	14.5	37.9	54.2	100
10% yeast	6	18.4	45.7	80.2	100
10% desiccated liver-N.F.	6	9.8	34.7(2)	67.7(2)	33.3

[1] The average initial body weight of rats in the various groups ranged between 45.4 and 46.8 gm.

[2] The alfalfa samples were kindly provided by the Research and Development Division of Nutrilite Products, Inc., Buena Park, California.

[3] Experimental period, 21 days.

[4] The values in parentheses indicate the number of animals alive at the time and on which averages are based. These values are given only for groups where the number per group differs from the number started.

[5] Solka Floc BW 200, Brown Co., Boston.

DISCUSSION

Present findings indicate that alfalfa contains a factor or factors apparently distinct from any of the known nutrients which counteracted the toxic effects of massive doses of Myrj 52, Tween 20 and Tween 60 in immature rats fed a highly purified low-fiber diet. The protective factor (or factors) is retained in the alfalfa residue fraction (the water-washed pulp remaining after the extraction of the juice). This is the same alfalfa fraction that partially counteracted the inhibitory effects of massive doses of estradiol on ovarian development in the immature rat (Ershoff et al., '56), prolonged the survival of immature hamsters fed highly purified diets (Ershoff, '56), promoted growth of immature guinea pigs fed a mineralized dried-milk ration (Ershoff, '57a) and counteracted the toxic effects of massive doses of glucoascorbic acid in the rat (Ershoff, '57b). It is also the same fraction that counteracted symptoms of mineral oil toxicity in rats and mice fed a low-fat ration (Ershoff and Hernandez, '58), prolonged the survival of hyperthyroid rats (Ershoff et al., '59) and counteracted symptoms of Tween 60 toxicity in the immature mouse (Ershoff and Hernandez, '59). The term "plant residue (PR) factor" has been suggested as a generic term for the substance (or substances) in alfalfa residue (and other succulent plants) responsible for the effects indicated above (Ershoff, '58).

In addition to alfalfa meal and alfalfa residue, rye grass, fescue grass, oat grass, orchard grass and wheat grass also had significant activity which was at least equal to that of alfalfa meal in counteracting Tween 60 toxicity in the immature rat. Carrageenin and sodium alginate were also active in this regard.

Significant activity in counteracting Tween 60 toxicity was also exhibited by cellulose per se. The protective effect of cellulose was proportional to the level fed; but at each level of feeding between 2.5 and 10% of the fiber its protective effect was less than that obtained with a comparable amount of alfalfa meal. Since the crude-fiber content of alfalfa meal is approximately 20%, a given weight of alfalfa meal supplies only one-fifth as much fiber as a comparable weight of cellulose. The increment in body weight obtained with a 5% alfalfa meal supplement was significantly greater, however, than that resulting from the addition of 1% of cellulose to the diet. It would appear, therefore, that the beneficial effects of alfalfa meal were due, at least in part, to some factor or factors other than its cellulose content per se; although the possibility has not been eliminated that cellulose, as present in alfalfa meal, may have growth-promoting activity not shared by other forms of cellulose.

No data are available as to the mechanism (or mechanisms) whereby alfalfa and other materials effective in counteracting Tween 60 toxicity exert their protective effect. Present findings, as well as those reported previously for the mouse (Ershoff and Hernandez, '59), indicate that the activity of the above materials is greater than can be accounted for on the basis of their bulk content alone, although the possibility exists that such materials provide residue which is particularly effective in counteracting surfactant agent toxicity, possibly by absorption of such surfactants in the gut. An alternate suggestion for the protective effect of various bulk-containing materials is that they are sources of an unidentified factor (or factors) which is required in increased amounts in animals fed massive doses of Tween 60 and other surfactant agents or stimulate the synthesis of such a factor(s) by the intestinal flora or the animals' own tissues (Chow et al., '53; Ershoff and Hernandez, '59).

SUMMARY

Immature rats were fed a highly purified low-fiber diet supplemented with massive doses of Myrj 45, Myrj 52, Tween 20, Tween 60 or Span 20, non-ionic surfactant agents with HLB (hydrophile-lipophile balance) values of 11.1, 16.9, 16.7, 14.9 and 8.6, respectively. Supplements of Myrj 45 at a 15 or 20% level in the diet resulted in a slight retardation in growth but had no deleterious effects on either gross appearance or survival during an experimental period of 21 days. Supplements of Myrj 52, Tween 20, Tween 60 or Span 20 at a 15 or 20% level in the diet, however, caused significant growth retardation, diar-

rhea, an unthrifty apearance and death. The deleterious effects of Myrj 52, Tween 20 and Tween 60 administration were completely counteracted by the concurrent feeding of alfalfa meal at a 20% level in the diet. Supplements of alfalfa meal were without protective effect, however, in rats fed diets containing Span 20. The protective factor (or factors) in alfalfa was retained in the alfalfa residue fraction (the water-washed pulp remaining after extraction of the juice). Supplements of protein, fat and the known vitamins and minerals were without protective effect. Purified cellulose, however, when incorporated in the diet had significant activity in counteracting the toxic effects of Myrj 52, Tween 20 and Tween 60 administration. The corrective effect of cellulose was proportional to the level fed; but at each level of feeding between 2.5 and 10% of the diet its protective effect was less than that of a comparable amount of alfalfa meal. In addition to alfalfa meal and alfalfa residue, rye grass, fescue grass, oat grass, orchard grass and wheat grass as well as carrageenin and sodium alginate also had significant activity in counteracting symptoms of Tween 60 toxicity in the immature rat.

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Supplementation of the Swine Gestation Diet with Pyridoxine[1]

H. D. RITCHIE, E. R. MILLER, D. E. ULLREY, J. A. HOEFER AND R. W. LUECKE

Departments of Animal Husbandry and Agricultural Chemistry, Michigan State University, East Lansing

Interest in vitamin B_6 metabolism during gestation was stimulated by reports of Wachstein and Gudaitis ('52a, '53) who found the existence of a relative pyridoxine deficiency in women during middle and late pregnancy, as measured by xanthurenic acid excretion. In earlier work with swine, Cartwright et al. ('44) reported that urinary excretion of xanthurenic acid is increased in pyridoxine-deficient pigs in amounts proportional to the level of tryptophan in the diet, and excretion of this metabolite ceases when sufficient pyridoxine is supplied. Moustgaard ('53) reported xanthurenic acid excretion to be a very sensitive test for pyridoxine deficiency in pigs. Chick et al. ('38), Hughes and Squibb ('42), Wintrobe et al. ('43), Lehrer et al. ('51), Moustgaard ('53) and Miller et al. ('57) have described various symptoms of pyridoxine deficiency in the suckling and the weanling pig. Draper et al. ('58) found no evidence of a derangement of tryptophan or pyridoxine metabolism associated with pregnancy in swine.

The purpose of the present study was to determine the effect of supplemental pyridoxine in the swine-gestation and lactation diet upon the performance of the sow and her offspring. The study was also designed to determine whether a relative vitamin B_6 deficiency exists in swine during some portion of the gestation period.

EXPERIMENTAL

Forty-four pregnant sows and gilts from the University herd were lotted equally into two treatments on the bases of size, sex, breed and litter. The control lot received a typical gestation ration (table 1) and the experimental lot received the same ration supplemented with 5 mg of pyridoxine·HCl per pound of diet. Upon analysis, using the chemical method of Fujita et al. ('55a, b, c), the control ration was found to contain 0.45 mg of total pyridoxine per pound (pyridoxine + pyridoxal + pyridoxamine). This level was shown previously by Miller et al. ('57) to be adequate to satisfy the vitamin B_6 requirement of the baby pig. Both groups of animals were hand-fed twice daily. The daily feed allowance was 5 pounds for gilts and 10 pounds for sows. All sows and gilts were weighed at two-week intervals.

TABLE 1

Composition of diet[1]

Ingredients	Per cent
Corn	58.0
Oats	20.0
Alfalfa meal	10.0
Soybean oil meal	7.0
Meat and bone scrap	3.0
Fishmeal	1.0
Dicalcium phosphate	0.5
Trace mineral salt	0.5
Vitamin B concentrate[2]	0.05
Vitamin A and D concentrate[3]	0.025

[1] Control ration contained 0.45 mg of pyridoxine per pound of diet. Experimental lot received same ration plus 5 mg of pyridoxine·HCl per pound.

[2] Contained in milligrams per pound: niacin, 20; Ca pantothenate, 20; riboflavin, 5; thiamine, 2.5; and vitamin B_{12}, 0.0045.

[3] Supplied 1000 I.U. of vitamin A and 300 I.U. of vitamin D per pound of diet.

Initially, each lot was composed of 13 first-litter gilts and 9 third-litter sows. However, it was necessary to remove two gilts from the experimental treatment because of failure to conceive. The trial commenced at a time when most of the ani-

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mals were in their second month of gestation and extended through lactation until each litter of pigs reached a standard weaning age of 35 days.

On two occasions, corresponding to middle and late pregnancy, a number of sows and gilts from both treatments were subjected to 48-hour confinement in metabolism cages to determine urinary excretion of xanthurenic acid, 4-pyridoxic acid and total creatinine before and after an oral dose of DL-tryptophan. Basal excretion of these metabolites was determined from collections taken during the first 24-hour period. Urine collections during the second 24-hour period were preceded by the tryptophan dosage, which amounted to 50 mg per kg of body weight. Xanthurenic acid was determined by the method of Wachstein and Gudaitis ('52b), 4-pyridoxic acid by the method described by Sarett ('51) and creatinine by the method of Clark and Thompson ('49).

Blood samples were drawn from the anterior vena cava at the end of each 48-hour period for red cell counts, white cell differentiation, hemoglobin, hematocrit and serum protein determinations. Total serum protein was determined by the biuret procedure accepted by the American Association of Clinical Chemists (Reiner, '53). Pooled porcine serum was standardized by macroKjeldahl methods. Electrophoretic separation of serum proteins was accomplished on a Spinco Model R paper electrophoresis system.[2]

Two gilts from the unsupplemented control lot received 400 mg of 2,4-dimethyl-3-hydroxy-5-hydroxymethylpyridine (desoxypyridoxine) daily in their diet during the final three weeks of gestation in an effort to produce a frank pyridoxine deficiency.

Shortly after birth, each pig was given an injection of an iron-dextran solution as a preventive measure against iron-deficiency anemia. All pigs were weighed at weekly intervals from birth through 5 weeks of age. Hemoglobin and hematocrit determinations were made on all pigs at birth and at one, three, and 5 weeks of age. In addition, red blood cell counts were taken on 5 litters in each treatment at these same intervals.

All data were treated statistically as outlined by Snedecor ('56).

RESULTS AND DISCUSSION

Table 2 shows a comparison of the productive performance of the two groups of sows and gilts during gestation and lactation. Performance of the pyridoxine-supplemented lot was slightly superior in respect to average daily gestation gain, total pigs per litter, live pigs per litter and total resorbed pigs. However, the differences are not statistically significant. The supplemented sows weaned an average of almost one pig more per litter than the controls; but the variation within lots was too great for any significance to be attached to this difference. Essentially no differences were observed between treatments for average litter weights at birth and at 35 days.

A summary of the urine studies at middle and late gestation is made in table 3. The higher xanthurenic acid excretion

[2] Beckman Instruments Inc., Spinco Division 1959 Instruction Manual, Model R Paper Electrophoresis System-RIM-5. Technical bull. 6026A.

TABLE 2

Effect of pyridoxine supplementation on performance of sows and gilts

	Treatment	
	Control	B_6-supplemented
Number of sows	9	9
Number of gilts	13	11
Av. daily gestation gain	0.85 ± 0.12[1]	1.00 ± 0.09
No. pigs born per litter	10.23 ± 0.72	10.65 ± 0.72
No. pigs born per litter (live)	9.55 ± 0.68	10.15 ± 0.70
Total number resorbed pigs	5	1
No. pigs weaned per litter	7.48 ± 0.49	8.30 ± 0.65
Litter weight at birth, pounds	29.1 ± 2.0	28.7 ± 2.0
Litter weight at 35 days, pounds	120.5 ± 9.9	125.2 ± 12.1

[1] Standard error of the mean.

TABLE 3

Effect of supplemental pyridoxine on urinology of sows and gilts at middle and late gestation[1]

	Treatment			
	Control		B_6-supplemented	
	Middle	Late	Middle	Late
Number of animals	12	20	12	19
Xanthurenic acid, mg/24 hours				
Before tryptophan	126 ± 14[2,3]	83 ± 12	179 ± 19[4]	96 ± 11
After tryptophan	134 ± 13[4]	79 ± 10	157 ± 27[3]	91 ± 11
4-Pyridoxic acid, mg/24 hours				
Before tryptophan	390 ± 46[4]	225 ± 27	654 ± 89[4]	343 ± 19
After tryptophan	502 ± 47[4]	271 ± 31	511 ± 61[3]	370 ± 33
Creatinine, gm/24 hours				
Before tryptophan	4.02 ± 0.39	3.51 ± 0.33	4.74 ± 0.38	4.22 ± 0.26
After tryptophan	4.07 ± 0.26	3.54 ± 0.23	3.94 ± 0.36	3.65 ± 0.20
Urine volume, ml/24 hours				
Before tryptophan	1984 ± 175	2172 ± 319	2637 ± 267	2171 ± 262
After tryptophan	2064 ± 183	1566 ± 124	2553 ± 214	1623 ± 144

[1] DL-tryptophan was administered in the diet at the end of the first 24-hour collection period at the rate of 50 mg per kg of body weight.
[2] Standard error of the mean.
[3] Significantly greater than corresponding value during late gestation ($P < 0.05$).
[4] Significantly greater than corresponding value during late gestation ($P < 0.01$).

rates of the experimental animals show that supplemental pyridoxine did not improve the efficiency of tryptophan metabolism over that of the controls. Neither lot showed a significant increase in xanthurenic acid excretion following the tryptophan load test. The control diet apparently contained enough pyridoxine to effectively metabolize the added tryptophan. Xanthurenic acid excretion was significantly higher at middle than at late gestation for each lot both before and after tryptophan dosage. The sows and gilts receiving added pyridoxine excreted appreciably greater amounts of 4-pyridoxic acid at both stages of gestation. More enlightening than this is the fact that in each 24-hour collection period, 4-pyridoxic acid excretion rates were significantly higher in midgestation than in late gestation for both lots. This observation, together with the differences noted for xanthurenic acid excretion, would lend support to the possibility that utilization of pyridoxine and tryptophan is more efficient in late gestation when the nutritional requirements of the developing fetus are greater.

It is also interesting to note that 4-pyridoxic acid was excreted in amounts considerably above the daily dietary intake of pyridoxine for both treatments. For example, daily pyridoxine intake of the supplemented sows was approximately 55 mg per sow, whereas daily excretion of 4-pyridoxic acid was consistently above 200 mg at both stages of gestation. This would seem to indicate there is considerable synthesis of pyridoxine by intestinal microorganisms in mature swine. Evidence of the synthesis of vitamin B_6 in man has been presented by Linkswiler et al. ('50). The creatinine excretion rate was determined in order to detect any differences in magnitude of tissue metabolism. Statistical analyses of the creatinine data did not reveal any significant differences with respect to either time or treatment.

Results of the hematological studies are presented in table 4. Pyridoxine supplementation had no significant effect on cellular or plasmal hematology during either stage of pregnancy. An anemia characterized by hypochromia and microcytosis has been observed in B_6-deficient pigs by numerous workers, notably, Hughes and Squibb ('42), Cartwright and Wintrobe ('44) and Miller et al. ('57). Lymphocytopenia has been noted in pyridoxine-deficient pigs (Miller et al., '57) and rats (Stoerk, '46; Agnew and Cook, '49; Din-

TABLE 4

Effect of supplemental pyridoxine on hematology of sows and gilts at middle and late gestation

	Treatment			
	Control		B_6-supplemented	
	Middle	Late	Middle	Late
Number of animals	12	20	12	19
Blood hemoglobin, gm/100 ml	14.1 ± 0.4[1]	12.8 ± 0.2	13.2 ± 0.3	13.1 ± 0.2
Hematocrit, % packed volume	40.1 ± 0.9	37.7 ± 0.5	39.2 ± 0.8	38.1 ± 0.5
Red blood cell count, millions/mm³	7.29 ± 0.26	6.58 ± 0.17	6.92 ± 0.19	6.79 ± 0.15
Reticulocytes[2]	0.68 ± 0.09	0.68 ± 0.09	0.67 ± 0.11	0.56 ± 0.10
Normoblasts[2]	0.03 ± 0.01	0.07 ± 0.01	0.03 ± 0.01	0.06 ± 0.02
Lymphocytes[3]	54.6 ± 3.3	49.2 ± 2.6	57.6 ± 1.9	50.7 ± 2.5
Monocytes[3]	2.0 ± 0.2	1.3 ± 0.1	2.0 ± 0.2	1.3 ± 0.2
Eosinophils[3]	5.7 ± 1.1	5.5 ± 0.7	5.9 ± 0.9	5.5 ± 0.5
Basophils[3]	1.0 ± 0.3	1.1 ± 0.1	1.3 ± 0.2	0.8 ± 0.2
Neutrophils[3]	36.8 ± 3.7	43.1 ± 2.5	33.4 ± 2.0	41.1 ± 2.9
Total serum protein, gm/100 ml	7.80 ± 0.20	7.51 ± 0.11	7.75 ± 0.11	7.55 ± 0.07
Albumin[4]	52.5 ± 1.4	51.2 ± 1.1	50.5 ± 1.9	52.9 ± 1.4
α Globulin[4]	17.2 ± 0.6	17.7 ± 0.7	18.6 ± 1.3	17.6 ± 0.6
β Globulin[4]	12.1 ± 0.5	14.2 ± 0.6	13.0 ± 1.0	12.8 ± 0.6
γ Globulin[4]	16.8 ± 1.9	16.9 ± 1.0	17.9 ± 1.2	16.7 ± 0.9

[1] Standard error of the mean.
[2] Expressed as a percentage of the total erythrocyte count.
[3] Expressed as a percentage of the total leukocyte count.
[4] Expressed as a percentage of the total serum protein.

ning and Day, '56). Such hematological derangements were not apparent in the unsupplemented swine used in the present study. Moustgaard ('53) has observed an impairment of the pyridoxine-deficient pig's ability to produce gamma globulin. In the present study, no such effect was noted in the unsupplemented lot and the mean values for gamma globulin are interpreted as being normal.

No improvement occurred in weight gain from birth to 35 days of pigs farrowed by the B_6-supplemented sows and gilts. In fact, a summary of weekly weights in table 5 reveals that offspring from the control sows and gilts were slightly heavier at every weighing and gained more rapidly during the 35-day lactation period than pigs from the supplemented animals. The fact that the latter group of sows farrowed and subsequently raised nearly one pig more per litter may account for these small differences.

A summary of hemoglobin, hematocrit and red blood cell values for the offspring at various ages is given in table 6. Pigs from the experimental sows and gilts had significantly higher values for these three

TABLE 5

Effect of supplemental pyridoxine on performance of offspring of swine during lactation

	Treatment	
	Control	B_6-supplemented
Number of pigs	225	213
Birth weight, pounds	2.85 ± 0.04[1]	2.69 ± 0.04
7-Day weight, pounds	5.13 ± 0.08	4.71 ± 0.02
14-Day weight, pounds	7.50 ± 0.15	7.01 ± 0.17
21-Day weight, pounds	10.11 ± 0.20	9.48 ± 0.23
28-Day weight, pounds	13.32 ± 0.21	12.30 ± 0.28
35-Day weight, pounds	16.10 ± 0.28	15.10 ± 0.35
Average daily gain, birth to 35 days, pounds	0.38 ± 0.01	0.36 ± 0.01

[1] Standard error of the mean.

TABLE 6

Effect of supplemental pyridoxine on hematology of offspring of swine during lactation

	Treatment	
	Control	B_6-supplemented
Blood hemoglobin, gm/100 ml		
Birth	11.1 ± 0.1[1]	11.8 ± 0.2[2]
7 Days	10.0 ± 0.1	10.0 ± 0.1
21 Days	10.3 ± 0.1	10.5 ± 0.2
35 Days	9.1 ± 0.2	9.3 ± 0.1
Hematocrit, % packed volume		
Birth	36.5 ± 0.4	39.4 ± 0.6[2]
7 Days	35.8 ± 0.4	34.9 ± 0.3
21 Days	35.7 ± 0.4	36.5 ± 0.3
35 days	32.8 ± 0.5	33.2 ± 0.5
Red blood cell count, millions/mm^3		
Birth	5.91 ± 0.15	6.75 ± 0.21[2]
7 Days	4.89 ± 0.29	4.87 ± 0.27
21 Days	5.75 ± 0.26	5.60 ± 0.22
35 Days	6.29 ± 0.29	5.64 ± 0.11

[1] Standard error of the mean.
[2] Significantly higher than the corresponding value for the controls ($P < 0.01$).

hematological measures at birth than the control pigs. During the remainder of lactation, such differences in the blood picture were not evident.

Nelson and Evans ('48, '51) have shown that the addition of the pyridoxine antagonist, desoxypyridoxine, to a pyridoxine-deficient diet resulted in marked reproductive upsets when adult female rats were placed on the diet 10 to 20 days prior to breeding. The incidence of resorptions was extremely high and survival of the young to weaning occurred rarely. In the present study, the following observations were made regarding the two gilts receiving 400 mg of desoxypyridoxine daily for three weeks prior to parturition: a marked diuresis was noted in both gilts, starting with the second week of desoxypyridoxine administration. In one 24-hour period each gilt excreted 5 liters of urine compared with an average excretion rate for the remaining gilts of about 2 liters. Xanthurenic acid excretion was elevated following an oral dose of DL-tryptophan; however, with the exception of one 24-hour period, the levels excreted were no higher than those of the other animals in the control lot. These excretion patterns resembled those reported in diabetic patients by Kotake and Tani ('53). Both gilts exhibited a greater than normal amount of distress and nervousness during parturition, but their pigs appeared strong and healthy at birth and grew normally throughout lactation. Neither gilt gave birth to any resorbed or stillborn pigs. The period of desoxypyridoxine administration was apparently too short to produce an acute deficiency.

Throughout this trial, no differences were observed in any of the criteria studied between gilts and sows in their response to the two dietary treatments.

SUMMARY

Forty-four pregnant swine were fed either a normal gestation ration or the same diet supplemented with 5 mg of pyridoxine·HCl per pound. Supplementation was begun during the second month of gestation and extended through a 35-day lactation period. Analyses of the data indicate that pyridoxine supplementation failed to improve the reproductive performance of the sow or the pre-weaning growth of her offspring. Furthermore, the additional pyridoxine had no significant effect upon the hematology or biochemistry of the sow during middle and late gestation. The 4-pyridoxic acid excretion rate in the urine of all sows in the trial greatly exceeded their dietary intake of pyridoxine. This suggests there may be appreciable bacterial synthesis of vitamin B_6 in the intestine of mature swine.

Pigs farrowed by pyridoxine-supplemented sows exhibited significantly higher hematological values at birth than those farrowed by unsupplemented sows; however, the differences vanished within a week following birth.

Daily administration of 400 mg of desoxypyridoxine in the diet of two gilts for three weeks prior to parturition failed to produce an acute deficiency, although chronic symptoms were observed.

Apparently the control diet contained enough pyridoxine (0.45 mg per pound of diet) to meet the swine gestation pyridoxine requirement for normal reproduction.

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Effects of Cholesterol and Other Substances on Essential Fatty Acid Deficiencies[1]

DAVID GAMBAL[2] AND F. W. QUACKENBUSH
Department of Biochemistry, Purdue University, Lafayette, Indiana

Cholesterol, cholic acid and bile salts, with or without certain vitamins, have recently been added to a number of experimental diets, especially in the study of atherosclerosis. That these compounds affect lipid metabolism is readily apparent from the work of several laboratories. Popjak ('46) reported that cholesterol addition to a diet increased mobilization of fat. Ridout et al. ('52) reported that it resulted in increased glyceride and cholesteryl esters of the liver. Alfin-Slater and co-workers ('54) reported similar results using low-fat diets. Cholate and bile salts were reported (Swell et al., '53) to increase blood cholesterol and perhaps alter fat metabolism indirectly through an effect on cholesterol. Vitamins reported to affect either cholesterol or liver lipids included choline (Moyer et al., '56), pantothenic acid, pyridoxine, riboflavin (Guehring et al., '52) and biotin (Okey et al., '51).

The purpose of this investigation was to study the effects of cholesterol, cholic acid, sodium glycocholate, and B vitamins and combinations thereof on the total carcass and liver lipids and on essential fatty acid- (EFA) deficiency symptoms of rats maintained on low-fat diets free from essential fatty acids.

EXPERIMENTAL

Male weanling rats of the Wistar strain, weighing from 40 to 50 gm were used in groups of 7 for dietary treatments of 5 weeks each. Additional animals were held in the negative control group for longer-term observations. The animals were housed in individual cages and fed the respective diets ad libitum for the 5 weeks. The percentage composition of the basal diet was as follows: glucose,[3] 74; fat-free casein,[4] 20; salts,[5] 4; celluflour,[6]. The vitamins were added in the following amounts in milligrams per kilogram of feed: thiamine·HCl, 20; riboflavin, 10; pyridoxine, 5; Ca pantothenate, 60; nicotinic acid, 25; *p*-aminobenzoic acid, 10; biotin, 2; inositol, 1200; folic acid, 10; vitamin B_{12} (0.1%) 1.8; choline, 1500; menadione, 50; α-tocopherol, 100. Vitamin A powder[7] was added at 2 I.U./gm of feed and vitamin D_3 at 0.2 I.U./gm of feed. The B vitamins were added as a premix with casein carrier, prepared in amounts to last one month. Hydrogenated coconut oil (iodine value, 2.5) was added to the completed diet at a 3% level except as otherwise stated. The diet and premix were stored at 0°C. In the positive control diet soybean oil replaced the hydrogenated coconut oil.

In the first series of experiments (table 1) 13 groups of rats received dietary supplements (1%) as follows: (1) positive control (3% soybean oil), (2) none (basal only), (3) cholesterol, (4) cholic acid, (5) cholesterol and cholic acid, (6) sodium glycocholate, (7) cholesterol and sodium glycocholate; (3a, 4a and 5a) in which 2% of a rice bran concentrate was added to diets 3, 4 and 5, respectively, and (3b, 4b and 5b) in which an additional 50% of the B-vitamin premix was added to diets 3a, 4 and 5, respectively. All of these diets except diet 1 contained 3% of hydrogenated coconut oil and no source of essential fatty acids.

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[1] Journal Paper no. 1257 of the Purdue University Agricultural Experiment Station.

[2] Present address: McCollum Pratt Institute, Johns Hopkins University, Baltimore.

[3] Cerelose, Corn Products Refining Company.

[4] Casein was extracted for three days with ether in a Lloyd's continuous extractor.

[5] McCollum Mix no. 185.

[6] Cellu Flour, Chicago Dietetic Supply House.

[7] Nutritional Biochemicals Corporation.

TABLE 1

Effects of supplements fed to weanling rats during a 5-week period

Diet no.	Supplement to basal diet[1]	Number of rats			Average gain in weight	Average total lipids in	
		Started	Developed dermatitis	Died		Carcass	Liver
					% of control	*% S.E.*[4]	*% S.E.*
1	Soybean oil (positive control)	5	0	0	100	4.9 ± 0.71	1.8 ± 0.10
2	None	7	0	0	75	4.4 ± 0.46	2.4 ± 0.78
3	Cholesterol	7	5	0	58	3.8 ± 0.57	3.4 ± 0.52
3a	Cholesterol and R.B.[2]	7	7	0	70	3.1 ± 0.28	3.8 ± 0.98[5]
3b	Cholesterol and R.B.[2] +B[2]	7	0	0	75	3.5 ± 0.41	5.0 ± 0.36
4	Cholic acid	7	0	2	36	1.6 ± 0.17	1.9 ± 0.35
4a	Cholic acid and R.B.[2]	7	0	0	45	1.5 ± 0.09	2.5 ± 0.19
4b	Cholic acid +B[2]	7	0	0	30	2.1 ± 0.22	3.9 ± 0.41
5	Cholesterol and cholic acid	7	0	0	59	1.7 ± 0.19	7.4 ± 1.01[5]
5a	Cholesterol, cholic acid, R.B.[2]	7	0	1	46	1.9 ± 0.12	12.9 ± 1.59[5]
5b	Cholesterol and cholic acid +B[2]	7	0	0	41	2.1 ± 0.23	9.4 ± 1.14[5]
6	Sodium glycocholate	7	3	0	66	2.7 ± 0.30	4.4 ± 0.52
7	Cholesterol and glycocholate	7	4	0	58	3.6 ± 0.24	13.7 ± 0.58

[1] All supplements at 1% of diet except as otherwise listed.

[2] B vitamins increased 50%.

[3] R.B. refers to 2% of water-soluble rice bran concentrate (Borden Co., Research Division).

[4] Standard error of the mean.

[5] Includes one atypical figure.

TABLE 2

Influence of saturated fat in EFA-deficient diet (5-week period; 7 rats/group)

Supplement to basal	Average gain in weight		Number showing dermatitis	
	No fat	3% fat[1]	No fat	3% fat[1]
	% of control	*% of control*		
None (control)	100	100	0	0
Cholic acid	41.2	48.0	0	0
Cholesterol	84.5	74.0	0	4
Cholesterol and cholic acid	48.6	79.0	0	1

[1] Hydrogenated coconut oil.

In the second series (table 2), diets 2, 3, 4 and 5 were repeated, with and without the hydrogenated coconut oil, to observe whether saturated fat is necessary for these compounds to exert their effects.

The experiments were terminated at the end of 5 weeks. The animals were weighed, examined for EFA-deficiency symptoms and killed with ether. All of the internal organs and skin were removed. The remaining carcass was weighed, cut into pieces and digested with 40% of NaOH (0.7 ml/gm of tissue) for two hours on the steam bath. The mixture was allowed to cool, 1/5th volume of ethanol was added and the heating continued for an additional hour. Capryl alcohol was added when needed to prevent foaming. When cool, the digest was acidified with hydrochloric acid (2:1), 20 ml of petroleum ether was added and the mixture filtered through Celite.[8] The residue was washed thoroughly with petroleum ether and the filtrate was extracted with 4 25 ml portions of petroleum ether. The ether layer was washed with water and dried over anhydrous sodium sulfate. The ether was evaporated under nitrogen and the residual lipid dried *in vacuo* at 50°C for one hour and weighed. The percentage of total lipid was based on the wet-carcass weight. The liver lipids were determined in a similar manner.

RESULTS

After 5 weeks, growth impairment as indicated by body weight was apparent with all diets fed except the positive control (soybean oil) diet. The weight range of the latter group at the end of the 5 weeks was 246 to 288 gm. The addition of cholesterol, cholic acid and sodium glycocholate retarded gains in weight, the maximum weights being only 58, 26 and 66% of the controls, respectively (table 1).

The addition of cholesterol and/or sodium glycocholate reduced the time required for the appearance of EFA-deficiency symptoms (5 weeks as compared with 8 to 10 weeks for animals fed the basal diet). The deficiency symptoms observed at 5 weeks were dermatitis, dandruff, dull hair coat and some loss of hair.

The most marked effects on liver lipids were obtained by the addition of cholic acid or sodium glycocholate to the cholesterol supplement. These substances increased the liver lipids three- to 4-fold over cholesterol alone. In all cholic acid-containing diets the carcass lipids were greatly reduced. Increasing the B vitamin content of the cholic acid diets tended to increase the liver lipids with a concomitant decrease in body weight.

The animals receiving cholic acid had a severe gastrointestinal disturbance, indicated by diarrhea, and two of them did not survive the 5-week period. In contrast with cholic acid the corresponding bile salt, sodium glycocholate, did not have such severe effects on the animals. The gastroenteritis and the retarding effect of cholic acid on growth were somewhat alleviated by the addition of cholesterol to the diet (diets 3 and 5). However, growth retardation by cholic acid seemed to be greater when the B vitamins were increased 50% or rice bran concentrate was added along with the cholesterol. In the absence of cholesterol the addition of the B vitamins with cholic acid had very little effect on weight, whereas the addition of rice bran concentrate appeared to relieve the in-

[8] Registered trade name for Johns Manville's diatomaceous silica filter aid.

testinal disturbance of the animals and resulted in increased food intake with subsequent weight gains.

The growth-retarding effect of cholesterol and cholic acid was also observed in EFA-deficient diets without fat (table 2). In this series control groups, the average weights of which were taken as 100%, both lacked EFA. The presence of hydrogenated coconut oil in the diet seemed to minimize the retarding effect of cholesterol-cholic acid combination on growth.

DISCUSSION

The manner in which the steroid substances retarded body weight is not clearly understood. However, the beneficial effects of additional B vitamins (50%) or of rice bran concentrate when given with cholesterol suggests the possibility that cholesterol may increase the requirements of certain B vitamins in EFA-free diets. On the other hand, cholic acid seemed able to nullify this effect of the B vitamins; indeed, it appeared as an antagonist. Growth retardation by cholic acid could not be attributed to decreased feed consumption as a result of gastrointestinal disturbance, since rice bran concentrate alleviated the latter but growth was still retarded.

In general, poor growth was accompanied by low carcass fat, as found in earlier studies (Quackenbush et al., '42). Cholesterol alone did not alter the carcass or liver fat substantially but increased the liver fat when fed with cholic acid and especially when fed with sodium glycoholate. Alfin-Slater et al. ('44) found that livers of EFA-deficient rats on a "fat-free" diet contained twice as much total lipid (6 to 7%) and twice as much total cholesterol as those of normal animals at the end of a 20-week period. Livers of our EFA-deficient animals which were fed cholesterol and glycocholate contained twice as much total lipid (13.7%) at the end of 5 weeks. Undoubtedly this effect of exogenous cholesterol was enhanced by the favorable conditions for cholesterol absorption which bile salt and 3% of dietary fat provided (Swell et al., '53). The cholesterol, once it is absorbed, may participate in esterification reactions (Daskalakis and Chaikoff, '55). Since the dietary choline level (0.15%) appears to be adequate for normal lipotropic activity (Ridout et al., '52), the high liver lipid appeared to be the result of interactions between EFA deficiency and absorbed cholesterol.

Whereas cholesterol and sodium glycocholate accelerated the appearance of EFA-deficiency symptoms on the EFA-free fat-containing diet, this effect was not observed on a completely fat-free diet. The time required for the skin symptoms to appear using the latter diet was 8 to 10 weeks for males and 12 to 15 weeks for females. This appears contrary to the findings of Peifer and Holman ('55) who reported accelerated depletion by cholesterol on fat-free diets and attributed the effect to an increased rate of utilization of EFA stores. Perhaps differences in diets and initial storage of EFA by the weanling rats are responsible for our different results. Essential fatty acid depletion in overall body stores is presumed when animals develop skin symptoms, since the lipids from a rat with EFA deficiencies were shown unable to cure the dermatitis (Quackenbush and Steenbock, '42). Total carcass lipids observed in our results, were not altered in most cases. The largest decrease occurred in cholic acid-fed rats but these showed no acceleration of EFA-deficiency symptoms. Therefore, cholic acid seems to have inhibited the formation or accelerated the utilization of body fat without affecting the EFA stores. If cholic acid inhibited the formation of fatty acids, perhaps it formed coenzyme A esters of cholic acid and thereby effectively reduced the synthesis of fatty acids, since coenzyme A esters of cholic acid have been reported by Siperstein and Murray ('56). Such specific action of cholic acid seems an attractive concept; however, it must be borne in mind that numerous factors in poor nutrition can limit total fat levels in the animal body (Quackenbush et al., '42).

SUMMARY

1. Cholesterol and sodium glycocholate accelerated EFA-deficiency symptoms using diets containing 3% of saturated fat; this effect was not observed on fat-free diets. Both substances retarded growth.

2. Cholic acid did not accelerate EFA-deficiency symptoms, but retarded growth and body-fat deposition and produced diar-

rhea. The diarrhea was alleviated by increased supplements of a rice bran concentrate but growth was not improved. Growth was improved by dietary cholesterol and fat.

3. When cholesterol was fed with either sodium glycocholate or cholic acid, the lipid content of the liver increased several fold. However, sodium glycocholate was the more effective of the two supplements, with cholesterol, to increase liver lipids and accelerate EFA-deficiency symptoms.

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Effect of Threonine Deficiency on Changes in Enzyme Activity and Liver Fat Deposition with Time[1]

CATHERINE CARROLL, DOROTHY ARATA AND DENA C. CEDERQUIST
Department of Foods and Nutrition, College of Home Economics, Michigan State University, East Lansing

All metabolic processes necessarily originate in cells. The increasing amount of research being done with cellular systems is providing some basic information about the actual biochemical reactions involved in the response of animals to environmental changes. Enzyme changes in rats have been observed in response to a threonine imbalance (Harper et al., '53). In this study, endogenous oxidation and the activities of the xanthine and tyrosine oxidase systems were decreased, whereas succinic and choline oxidase activities were increased to some extent. An investigation of similar enzyme changes with concurrent accumulation of liver fat in rats subjected to amino acid imbalance has yielded information about the complicated interrelationships between these two factors. Namely, when a double amino acid deficiency was produced in rats by restricting tryptophan as well as threonine, the enzyme changes observed when using the threonine-deficient diet did not occur, but fat accumulated in the livers to the same extent as on the single deficiency (Arata et al., '54).

The study reported in this paper was undertaken to determine the nature of the biochemical lesions induced by a deficiency of threonine superimposed on a low protein diet. The activities of several enzyme systems were used as criteria for the metabolic activity of liver tissues. The study was divided into two parts. Experiment 1 was designed to follow the development of biochemical lesions as a function of time. This approach was chosen in order to determine (1) which enzyme systems would be affected and (2) at what point in time maximum changes would occur. Experiment 2 involved the use of a larger number of rats and was designed to collect data pertaining to enzyme activities and liver fat levels at the time of maximum changes observed in experiment 1, and also 4 weeks later.

EXPERIMENTAL

Male weanling rats of the Sprague-Dawley strain were used in the two experiments. In experiment 1, three groups of 14 to 20 animals each were fed the experimental diets ad libitum for two to 36 days. Group 1 received a 25% casein ration and served as the primary control group. This ration was composed of casein, 25; choline, 0.15; salts W[2], 4.0; vitamin mix[3], 0.25; corn oil[4], 4.0; cod liver oil, 1.0; and sucrose to make 100 gm. Rats in group 2 were fed a 9% casein ration supplemented with 0.36% DL-threonine; 0.30% DL-methionine; and 0.10% DL-tryptophan, and served as the secondary control group. Other constitutents of this diet were identical with those in the diet for group 1; the weight difference was adjusted with sucrose. The diet for group 3 was identical with that for group 2 except that no threonine was added to this ration.

After two, 7, 12, 19, 24, 31 and 36 days on the diets, rats were stunned by a sharp blow on the head and decapitated. The

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[1] Journal article 2505 from the Michigan Agricultural Experiment Station.

[2] Obtained from Nutritional Biochemicals Corporation.

[3] The vitamin mix provided the following in milligrams/100 gm of diet: thiamin, 0.5; riboflavin, 0.5; niacin, 1.0; pyridoxine, 0.25; Ca pantothenate, 2.0; inositol, 10.0; folic acid, 0.02; vitamin B_{12}, 0.002; and biotin, 0.01.

[4] Containing 7.5 mg α-tocopheryl acetate and 0.375 mg menadione.

livers were removed as rapidly as possible, chilled for a few seconds in crushed ice, blotted free of excess moisture, weighed, and then homogenized in cold sodium potassium phosphate buffer, pH 7.3. A portion of the homogenate was used for enzyme determinations. The remainder of the homogenates were stored in the cold and later analyzed for fat.

Endogenous oxidation and xanthine oxidase (Axelrod and Elvehjem, '41), succinic dehydrogenase (Umbreit et al., '51) and malic dehydrogenase (Potter, '46) were measured by manometric procedures using the Warburg apparatus. The xanthine oxidase method was modified by using only 1 ml of 16.7% liver homogenate in the reaction mixture. The method for malic dehydrogenase was modified to the extent that livers were homogenized in 0.039 M sodium potassium phosphate buffer rather than in water.

All flasks were incubated at 37° and allowed to equilibrate for 10 minutes. Substrates were tipped in from the side arms, and readings were taken at 10-minute intervals for the first three systems, and at 5-minute intervals for malic dehydrogenase. Cytochrome C preparations were isolated from beef hearts according to the method of Keilin and Hartree ('45).

In addition to the enzyme systems mentioned, the rat livers were analyzed for fat. The homogenates remaining after the aliquots were taken for enzyme determinations were transferred to evaporating dishes and dried at 90° for 12 hours. The dried livers were then ground and the percentage of fat determined by ether extraction on one-gram samples.

In experiment 2, similar groups and diets were used, except that vitamin A and D concentrates[3] were used instead of cod liver oil in the rations. In each group 11 of the 22 rats were fed ad libitum for 15 days and the remainder for 43 days. The rats were killed at the close of these two periods and endogenous oxidation and the activities of the xanthine oxidase and malic dehydrogenase enzyme systems were measured.

Livers were analyzed for fat and nitrogen at 15 days and again at 43 days. Nitrogen was determined by the macro-Kjeldahl method on 300 mg samples of the dried, ground liver from which the fat had been extracted.

RESULTS

Livers taken from rats in group 1 (25% casein) at 15 days contained 2.6 ± 0.1 gm of nitrogen/100 gm of liver tissue, and at 43 days 2.9 ± 0.1 gm of nitrogen. The percentage of nitrogen in livers from rats in groups 2 and 3 was significantly lower at 15 days, namely 2.1 ± 0.1 gm for group 2 and 2.0 ± 0.1 gm for group 3. At the end of 43 days, liver nitrogen values were 2.5 ± 0.1 and 2.4 ± 0.1 gm/100 gm of liver for groups 2 and 3, respectively.

The fat content of rat livers in experiments 1 and 2 are presented in table 1 for groups 1, 2, and 3 for each time period. The average percentage of liver fat in rats fed the 25% casein diet (group 1) remained fairly constant, ranging from 5.1 to 7.9% in experiment 1. A slightly higher fat content was noted in livers of rats fed the 9% casein ration supplemented with threonine (group 2), a range of 7.3 to 13.8%. In group 3 (9% casein with no added threonine) there was a progressive increase in liver fat to 30.4% on the 24th day, after which time the level decreased steadily to 17.4% by the 36th day and finally to 14.7% on the 43rd day.

Enzyme activities for group 1 (25% casein) and group 2 (9% casein plus threonine) approached similar values when calculated on a per-gram-of-nitrogen basis, e.g., at 15 days the endogenous oxidation in group 2 was 110% of that in group 1. The corresponding value for the activity of xanthine oxidase was 79%, and of malic dehydrogenase 91%. Inasmuch as no significant differences in activity could be observed between groups 1 and 2 in any enzyme system studied except xanthine oxidase, group 2 was chosen as the primary control in interpretation of these data. In addition, since no differences were observed in nitrogen content of the rat livers in group 2 when compared with those from group 3, enzyme activities are reported in terms of grams of liver tissue rather than grams of nitrogen (table 1).

[3] Providing 200 I. U. vitamin A and 150 units vitamin D/100 gm diet.

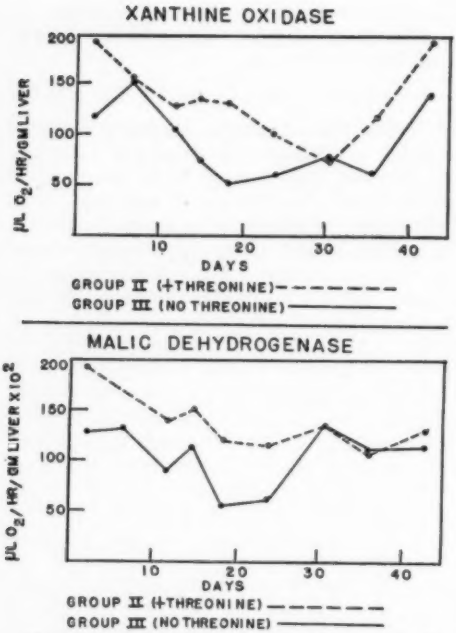

Fig. 1 Activities of xanthine oxidase and malic dehydrogenase plotted against time for rats fed 9% casein with threonine (group 2) and without threonine (group 3).

When the enzyme activities per gram of liver in groups 2 and 3 are plotted against time, a relationship can be observed between time and the activities of two of the enzyme systems, xanthine oxidase and malic dehydrogenase (fig. 1). In each of these systems, the greatest differences between groups 2 and 3 occurred at about the 19th day of the experiment. With the use of a larger number of rats in each group, statistically significant differences were observed between groups 2 and 3 for both systems on the 15th day (table 1). On the 43rd day, these differences had decreased in the case of xanthine oxidase and disappeared entirely in the malic dehydrogenase system (table 1).

The data collected for succinic oxidase were inconclusive. There was sufficient variation within a group to negate any difference that might have existed between groups, and therefore no statistically significant comparisons can be made.

DISCUSSION

The activities of the xanthine oxidase and malic dehydrogenase systems in the control group (group 2) decreased with time (fig. 1). The degree to which this decline in activity was manifested varied with the system under study. In the case of malic dehydrogenase, the decline was gradual and the curve tended to plateau around the 20th day of the experiment. In the 43-day period, the activity of this system decreased approximately 65%.

TABLE 1

Enzyme activities in rats fed 9% casein diets with (group 2) and without (group 3) added threonine

Days on diet	No. rats	Liver fat[1]		Xanthine oxidase[2]		Malic dehydrogenase[2]	
		Group 2	Group 3	Group 2	Group 3	Group 2	Group 3
				Experiment 1			
2	3–5	7.3	7.0	193	117	194	130
7	2–4	13.6	21.7	156	150	—	135
12	2–4	12.9	20.8	129	108	141	90
19	2	11.5	22.6	135	51	123	57
24	2	10.2	30.4	102	64	120	63
31	2	13.8	18.6	75	76	138	138
36	2	8.3	17.4	120	66	108	111
				Experiment 2			
15	11	11.4 ± 1.3[4]	23.0 ± 1.1	136 ± 9	76 ± 9	151 ± 6	115 ± 10
43	11	8.6 ± 0.3	14.7 ± 0.8	197 ± 16	145 ± 13	133 ± 14	111 ± 34

[1] Expressed as percentage of dry weight of liver.
[2] Expressed as μl O_2/hour/gm liver.
[3] Expressed as μl O_2/hour/10 mg liver.
[4] Standard error of the mean.

Xanthine oxidase in the control group (group 2) described a pattern different from malic dehydrogenase. The rate at which xanthine was oxidized by the tissue homogenates fell precipitously with time. The activity of this enzyme system fell steadily for the first 31 days of the experiment, until the activity was only 38% of the initial rate measured on the second day of the experiment. Unlike malic dehydrogenase, the activity curve for xanthine oxidase did not attain a state of equilibrium. A period of recovery of this system began on the 31st day. At the close of the experimental period, the activity of the xanthine oxidase system in the control group was identical with that measured at the start of the experiment.

These patterns of enzyme activity plotted against time varied with a change in diet. When rats were fed a 9% casein diet not supplemented with threonine (group 3), the decline in enzyme activity was more rapid and more severe than observed in the control rats. In the threonine deficient animals, the period of decline in enzyme activity was followed by a period of recovery. The recovery of the malic dehydrogenase system was complete by the 31st day of the experiment. No significant differences between the deficient group and the control group were observed from the 31st day to the close of the study. The recovery of the xanthine oxidase system was incomplete. On the 43rd day of the experiment the activity of this enzyme in the threonine deficient group was still below that in the control group.

These observations suggest the importance of choosing the proper point in time to observe maximum differences between groups when enzyme systems are to be used as assay tools. A time study is strongly indicated as a preliminary approach to this type of study.

When xanthine oxidase and malic dehydrogenase activities of group 3 (threonine deficient) are expressed as percentage of the control (group 2), and superimposed on a bar graph showing percentage of fat in livers from threonine deficient rats, the temporal relationships between enzyme activity and liver fat deposition become more evident (fig. 2). The fat

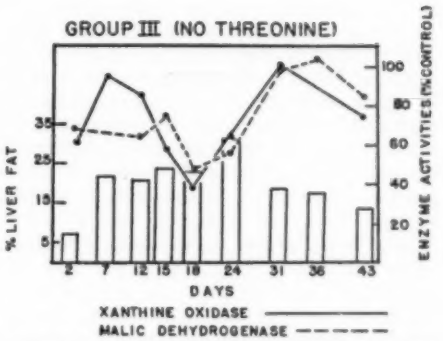

Fig. 2 Enzyme activities for group 3 (no threonine) plotted against time and superimposed on bar graph of liver fat levels. Enzyme data are expressed as percentage of control (9% of casein supplemented with 0.36% of DL-threonine).

was not mobilized out of the livers of the threonine deficient rats until *after* the enzymes had begun to recover. A similar observation was made by Arata et al. ('56) when they observed maximum stimulation of endogenous oxidation by added diphosphopyridine nucleotide (DPN) in livers of threonine deficient rats before the peak in liver fat deposition.

An interesting observation made during the course of measuring xanthine oxidase in the study reported in this paper led to a consideration of the possible involvement of DPN in this system. The time required for liver homogenates to begin oxidizing xanthine was considerably longer in the threonine deficient group, namely, an average of 133 minutes for group 3 as compared with 77 minutes for group 2. This time factor was more marked at 15 days than at 43 days, when differences in enzyme activity between groups were smaller. It was apparent that some factor (or factors) other than the concentration of the apoenzyme was responsible for the changes in activity observed in this system. In studies with deuterium-labeled substrates, Vennesland ('56) showed xanthine oxidase catalyzed the reduction of DPN. This observation could implicate DPN as a coenzyme for xanthine oxidase despite the unfavorable oxidation-reduction potentials. The deficiency of this coenzyme observed in threonine deficient rats (Arata et al., '56) could have been limiting the rate at

which xanthine oxidase oxidized the substrate in the experiments reported here. This possibility was investigated by the addition of DPN *in vitro* to xanthine oxidase flasks. The addition of DPN caused a stimulation of xanthine oxidase activity in liver homogenates from threonine deficient rats, but the results were variable and not proportional to the decreased activity of this system observed without added DPN. Thus, the metabolic defect observed in this system is more complex than a simple deficiency of DPN.

In addition to biochemical changes, physical alterations in particulate cellular material may also play a role. For example, Dianzani ('55) reported increased permeability of the mitochondrial membrane in his studies of fatty livers produced by means other than an amino acid imbalance.

Further work is in progress to investigate the interrelationships between liver fat and selected enzyme and coenzyme systems.

SUMMARY

Albino rats were fed a low protein diet (9% casein) deficient in threonine. The activities of several enzyme systems were measured at intervals over a period of 43 days. Control rats were fed the same diet supplemented with 0.36% of DL-threonine.

The deposition of liver fat in the threonine deficient rats reached a peak in 24 days. After 6 weeks the level of fat in the livers of these rats had fallen to approximately half of this maximum.

The activities of two enzyme systems, xanthine oxidase and malic dehydrogenase, varied with time. The maximum decrease in activity of these systems in the threonine deficient group, as compared with the control, occurred on the 19th day of the experiment. This phase of decreasing activity was followed by a period of recovery.

The fat was not mobilized out of the livers of the threonine deficient rats until *after* the enzymes had begun to recover.

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Response of Human Beings to a Low-Vitamin B_6 Diet[1,2]

KATHERINE E. CHESLOCK AND MARGARET T. McCULLY
Nutrition Laboratory, School of Home Economics, University of Delaware, Newark

Recent findings indicate that vitamin B_6 deficiency in human beings may be more prevalent than heretofore recognized (Hunt, '57; Turner and Reynolds, '55). Vilter et al. ('53) demonstrated that a deficiency of the vitamin, induced by administering the antimetabolite, deoxypyridoxine, mimics symptoms of deficiencies of other B vitamins. It would be expedient if a deficiency of vitamin B_6 could be detected before latent critical symptoms appear.

Few tests have proven to be useful in evaluating nutritional status in human beings with regard to vitamin B_6. Xanthurenic acid excretion in the urine of human beings who have received a dose of tryptophan is increased when the requirement for vitamin B_6 is abnormally high[3] (Wachstein and Gudaitis, '52). Lymphopenia was noted in human subjects when a deficiency was induced with deoxypyridoxine (Vilter et al., '53). Tissue concentration of vitamin B_6 may be expected to decrease in human beings with an inadequate intake of the vitamin as this was shown to occur in the tissues of rats (Cheslock, '58).

Reported herein are results of an investigation on human beings who consumed a diet low in vitamin B_6. Vitamin B_6 content of the blood, hemoglobin and formed elements in the blood, and xanthurenic acid excretion after a test dose of tryptophan were measured. Nitrogen balance was determined for three periods during the experiment.

EXPERIMENTAL PROCEDURES

Eight college students, 7 young women and one young man, ages 18 to 20, served as subjects for the experiment. They were given physical and hematological examinations before the experiment began and judged to be in good health. A physician made a brief examination every other week during the experiment. This examination consisted of an exercise tolerance test following a technique similar to that described by Dr. W. H. Forbes of the Harvard Fatigue Laboratory (Thorndike, '56), and an inspection of the lips, mucous membranes of the mouth and the skin. No signs of deficiencies were apparent during the 7 weeks, except the complaint of fatigue in three subjects which is not infrequent for college students. A record of the weight of each subject was kept.

The subjects were maintained for 52 days on a diet which was low in vitamin B_6. The 4-day diet pattern used, with amounts of each food, is given in table 1. The diet consisted chiefly of natural foods which were selected because they were found by determination to be low in vitamin B_6. The content of vitamin B_6 in 47 foods is shown in table 2. This analysis for vitamin B_6 was performed as previously described (Cheslock, '58). The diet for the women was found by periodic analysis of aliquots of the meals to provide an average of 0.414 mg of vitamin B_6 and 4.22 gm of nitrogen per day. The man received additional bread and meat and his diet provided an average of 0.50 mg of vitamin B_6 and 5.75 gm of nitrogen per day. A supplement containing 0.7 mg each of

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[2] This research was supported in part by a grant from the University of Delaware Research Foundation.

[3] Bessey, O. A., D. J. D. Adams, D. R. Bussey and A. E. Hansen 1954 Vitamin B_6 requirements of infants. Federation Proc., *13:* 451 (abstract).

TABLE 1

Menus for low vitamin B_6 diet

Day 1	gm	Day 2	gm	Day 3	gm	Day 4	gm
			Breakfast				
Grapefruit juice	100	Grapefruit juice	100	Orange juice	100	Grapefruit juice	100
Puffed rice	14	Egg	54	Cream of Wheat	120	Egg	54
Toast	23	Toast	23	Toast	23	Toast	23
Butter	5	Butter	5	Butter	5	Butter	5
Jelly	15	Jelly	15	Jelly	15	Jelly	15
Milk	120	Coffee	—	Milk	120	Coffee	—
Sugar (to taste)	—	Sugar	—	Sugar	—	Sugar	—
			Lunch				
Egg	54	Carrots	100	Egg	54	Carrots	100
Lettuce	25	Beets	75	Lettuce	25	Beets	75
Celery	12	Asparagus	75	Celery	12	Asparagus	75
Carrots	25	Biscuit	38	Tomato	25	Biscuit	38
Bread	23	Butter	5	French dressing	—	Butter	5
Jelly	15	Jelly	15	Biscuit	38	Jelly	15
Butter	5	Peaches	100	Butter	5	Peaches	100
Pears	100	Milk	120	Jelly	15	Milk	120
				Lemonade	110		
				Apple	100		
			Dinner				
Frankfurter	51	Hamburger	50	Frankfurter	51	Hamburger	50
Peas	65	Tomato	75	Noodles	100	Tomato	75
Rice	100	Macaroni	100	Butter	5	Spaghetti	100
Butter	10	Lettuce	25	Green beans	75	Lettuce	25
Strawberries	100	Dressing	—	Peaches	100	Dressing	—
with cream	30	Pears	100	Milk	120	Pears	100
with marshmallows	—	Ice cream	81	Cake	75	Ice cream	81
Milk	120						

TABLE 2

Vitamin B_6 content of foods as analyzed by modified S. carlsbergensis assay

TABLE 2

Vitamin B_6 content of foods as analyzed by modified S. carlsbergensis assay

Cereals and starches		Fruits and vegetables		Meats and Fish		Milk and beverages		Miscellaneous	
	mg/100 gm		*mg/100 gm*		*mg/100 gm*		*mg/100 gm*		*mg/100 gm*
Puffed rice dry weight	0.0700	Peaches canned	0.0146	Frankfurter raw	0.1831	Lemonade frozen	0.0154	Vegetable beef soup frozen	0.0697
Rice Krispies dry weight	0.1351	Pears canned	0.0191	Frankfurter cooked	0.1645	Milk, liquid homogenized	0.291	Chicken noodle soup condensed	0.0521
Special K dry weight	0.0643	Applesauce canned	0.0315	Ground beef raw	0.241	Powdered milk dry weight	5.43	Scotch barley broth condensed	0.0727
Wheat Chex dry weight	0.1074	Rhubarb frozen	0.0109	Ground beef broiled	0.0726	Milk canned	7.5	Chicken vegetable soup condensed	0.0437
Wheaties dry weight	0.0643	Raspberries frozen	0.0188	Ham slice broiled	0.3569	Milk, low-sodium	1.479	Tomtao soup condensed	0.0428
Cream of Wheat cooked weight	0.0468	Strawberries frozen	0.0104	Salmon canned	0.282	Coca cola	0.27	Pickle	0.0684
Minute Rice dry weight	0.0348	Peas frozen	0.1145			Coffee percolated	0.0028		
Instant potato dry weight	0.4676	Green beans frozen	0.0307			Bouillon	0.060		
Saltines dry weight	0.0683	Grapefruit juice canned	0.006						
Bread dry weight	0.0539	Asparagus canned	0.0554						
Macaroni cooked weight	0.0124	Tomato canned	0.0475						
Spaghetti cooked weight	0.0376	Carrots canned	0.0172						
Egg noodles cooked weight	0.0199	Beets canned	0.0278						

vitamin B_1, B_2 and niacin and 7 mg of iron was given to each subject daily because the diet was calculated to contain less than three fourths of the National Research Council's recommended allowances for these subjects.

The need for energy and protein was estimated in a 12-day pilot study which preceded the experiment. Nitrogen balance was determined to establish adequacy of the diet with respect to protein, and additional food was given when the subjects expressed hunger. The basal diet supplied 2045 and 2724 Cal. for the women and the man, respectively. Additional energy was supplied in the form of soft drinks, candy, butter and/or jelly according to need.

Four times during the experiment blood was drawn early in the afternoon from the antecubital vein for hematological examination and analysis of vitamin B_6. Analysis of vitamin B_6 was performed as previously described (Cheslock, '58). Red and white cell counts, hemoglobin and differential determination were made by standard techniques.

Five grams of L-tryptophan were given to each subject at the beginning of the experiment and during the 4th and 7th weeks. It was taken orally at 5 P.M. the evening preceding the day on which (at approximately 7 A.M.) complete urine collections were begun for xanthurenic acid determination. Tryptophan was taken on the day on which blood was drawn for analysis of vitamin B_6, but consumed after the blood sample was taken. Xanthurenic acid was determined according to the method of Rosen et al. ('51).

At the termination of the experiment, a 100 mg dose of pyridoxine hydrochloride in water was given at 10 P.M. and blood was drawn the next afternoon for analysis of vitamin B_6. Blood samples were also obtained 8 weeks after the close of the experiment for vitamin B_6 analysis.

Complete collections of urine and feces were made for three 4-day periods during the study. Nitrogen balance was determined after analysis by the Kjeldahl method.

TABLE 3

Results of blood analysis

Subjects	Zero time		4 weeks		7 weeks		3/27/58[1,2]		5/27/58[3]
	Vitamin B_6 content[1]	Absolute lymphocyte count	Vitamin B_6 content	Absolute lymphocyte count	Vitamin B_6 content	Absolute lymphocyte count	Vitamin B_6 content	Absolute lymphocyte count	Vitamin B_6 content
	μg/100 ml[4]	*mm*[3]	*μg/100 ml*[4]	*mm*[3]	*μg/100 ml*[4]	*mm*[3]	*μg/100 ml*[4]	*mm*[3]	*μg/100 ml*[4]
BF	0.59	4,004	0	1,911	0	2,288	5.54	2,772	1.28
GW	0.89	2,920	0	2,610	0	—	4.33	2,662	2.10
AH	1.09	2,222	0	2,613	0	3,344	6.00	2,140	1.35
TR	0.87	3,802	0	2,635	0	3,264	5.74	3,696	1.81
NS	1.32	2,740	0	2,090	0	2,550	5.68	2,882	2.46
MW	1.17	2,472	0	1,890	0	1,518	6.91	1,701	2.28
NL	1.78	5,240	0	5,376	0	5,380	5.03	5,095	14.94[5]
SW	—	2,534	0	3,296	0	1,980	3.03	1,809	—

[1] All subjects were given 100 mg of pyridoxine·HCl on 3/26/58.
[2] A significant difference occurs between zero time and 7 weeks, $t_{(6.05)} = 2.42$.
[3] These values were obtained 8 weeks after termination of the experimental diet.
[4] Standard error of the mean: ± 0.15.
[5] This subject had undergone an operation for removal of a thymoma during the preceding month.

RESULTS AND DISCUSSION

The results of the analyses of vitamin B_6 of the blood are presented in table 3. The content of the vitamin in the blood fell to zero within 4 weeks and remained there until the supplement of pyridoxine was given. Each subject was given a supplement of 100 mg of pyridoxine hydrochloride on March 26 and the blood concentration of the vitamin was significantly higher on March 27 than for any other time of analysis.

The blood concentration at zero time represents values for individuals on self-selected diets. They were lower than values obtained 8 weeks after completion of the experimental diet and lower than values obtained from an older group of subjects who had been consuming self-selected diets (see below). The experimental period was preceded by an academic final-examination period for these students and this may account for the low blood concentrations at zero time.

Before the experiment began, a pilot study was conducted to determine whether the blood content of vitamin B_6 could be increased by giving a supplement of the vitamin. Four persons, ages 27 to 37, who were consuming self-selected diets, showed values of 1.28, 1.65, 2.17 and 2.55 μg of vitamin B_6 per 100 ml of blood. After receiving a supplement of 5 mg of pyridoxine hydrochloride per subject per day for 28 days, the concentration of vitamin B_6 in the blood of these subjects was 7.10, 5.26, 5.44 and 7.08 μg/100 ml, respectively. Marsh et al. ('55) reported an increase in blood level of pyridoxine when a supplement of vitamin B_6 was given to human subjects daily. Normal values for their subjects on self-selected diets were from 2 to 4 μg/100 ml of blood. These values were increased to 8 to 10 μg after supplementation with 10 to 15 mg of pyridoxine for 4 or 6 weeks.

In table 3 is recorded the absolute lymphocyte count of the blood of the experimental subjects taken on the same day as blood was drawn for analysis of vitamin B_6. Decreases in lymphocyte counts during the 7 weeks were observed in 5 of the 8 subjects. Subject NL, who had the highest lymphocyte counts throughout the study, had a thymoma removed the month after the experiment ended. Two subjects had slight infections during the study. Subject SW reported a dental infection on February 25 and GW had an upper respiratory infection and was retained in the student infirmary between February 14 and 17. The change in lymphocyte counts between the zero time and 7th week is significant at the 5% level. This is in agreement with the findings of Hawkins and Evans ('52) who developed lymphopenia in white rats and dogs deprived of vitamin B_6. Vilter et al. ('53) reported lymphopenia as the only significant hematological finding when a deficiency of the vitamin was induced with deoxypyridoxine in human beings. The evidence indicates that vitamin B_6 may have some role in the maturation of lymphocytes.

No consistent differences were noted for white blood cell counts, red blood cell counts and other cellular components of the differential determination. All subjects demonstrated an increase in hemoglobin concentration of the blood while on the experimental diet. This may have been due to the fact that they were receiving a supplement of iron.

Values for xanthurenic acid excretion of the subjects are presented in table 4. The analysis was made on a 24-hour urine collection beginning at approximately 7 A.M the day following the administration of 5 gm of L-tryptophan, and is presented as milligrams per day. An increase in the excretion of xanthurenic acid was noted for all subjects except one. Five subjects excreted more than 30 mg per day, the amount which Vilter et al. ('53) considered to be the figure above which a deficiency of vitamin B_6 may be thought to exist. Two subjects (TR and NS) who excreted less than 30 mg of xanthurenic acid also exhibited little change in the lymphocyte count. The male subject, SW, also did not show much change in xanthurenic acid excretion, but experienced a decrease in lymphocyte count. He had received more vitamin B_6 than the women. Two explanations could be offered for the lack of response to the administration of tryptophan by these subjects; either the amount of vitamin B_6 in the diet was sufficient, or

* Standard error of the mean: ± 0.15.
* This subject had undergone an operation for removal of a thymoma during the preceding month.

TABLE 4

Results of analysis of urine and feces

Subjects	Weight	Zero time	2 weeks	4 weeks		6 weeks	7 weeks
		Xanthurenic acid[1]	Nitrogen[2]	Xanthurenic acid[1]	Nitrogen[2]	Nitrogen[2]	Xanthurenic acid[1]
		mg/day	*gm/4 days*	*mg/day*	*gm/4 days*	*gm/4 days*	*mg/day*
BF	124	7.7	−0.50	31.8	+0.40	+0.39	51.3
GW	138	31.8	−1.16	180.7	+0.66	+2.07	226.8
AH	118	42.3	−2.30	58.5	−1.58	+0.43	73.5
TR	110	10.8	−0.99	38.0	−1.60	+0.29	16.4
MW	110	14.6	−2.91	18.6	−4.20	−0.12	20.2
NS	135	21.5	+0.47	35.7	−0.06	−0.74	46.4
NL	136	9.4	−1.57	5.1	−4.03	−1.90	41.6
SW	136	10.2	+1.19	7.6	−0.32	+3.67	25.1

[1] Xanthurenic acid excreted.
[2] Grams nitrogen retained or lost for a 4-day period.

intestinal synthesis provided the vitamin. It is interesting that the blood content of vitamin B_6 of all of the subjects responded by falling to zero after 4 weeks, but the xanthurenic acid excretion of three subjects did not rise above 30 mg. These three subjects may have been receiving sufficient vitamin B_6 to maintain some of their needs, but not their blood levels. These results were analogous to those obtained using the albino rat. In studies conducted on rats (Cheslock, '58), 10 μg of vitamin B_6 in the diet maintained growth; however, 20 μg of the vitamin was necessary in order to detect vitamin B_6 in the blood when the animal was fed ad libitum.

Complete collections and analysis of urine and feces were made during the first, 4th and 7th weeks of the experiment for 4-day periods. Nitrogen balance was determined for these periods and the data are presented in table 4 with the weights of the subjects. No significant change in body weights occurred during the experiment. Two months before the experiment began, 7 of the subjects were maintained in nitrogen balance for 12 days when consuming the same amount of nitrogen as during the experiment. The subjects could lose nitrogen without losing weight. The subject, NL, who had the thymoma removed, consistently lost considerable nitrogen.

SUMMARY

Eight college students were maintained for 52 days on a diet which was low in vitamin B_6. Blood content of vitamin B_6 dropped to zero within 4 weeks and remained there until a 100 mg supplement of pyridoxine hydrochloride was given. Lymphocyte counts decreased in 5 subjects. Xanthurenic acid excretion, after a test dose of tryptophan, increased to greater than 30 mg per day for 5 of the subjects. No dermal symptoms were evident. The use of lymphocyte counts along with the test dose of tryptophan may prove useful in experimental studies to establish the requirements for vitamin B_6. Results of the present study indicate that the requirement for young college women is above 0.5 mg per day.

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Effect of Excess Dietary Zinc on Iron and Copper in the Rat

DENNIS H. COX AND DOROTHY L. HARRIS
Department of Animal Diseases,
Georgia Coastal Plain Experiment Station,
Tifton

Zinc toxicity in the rat is manifested by a hypochromic, microcytic anemia (Sutton and Nelson, '37; Smith and Larson, '46), poor growth (Smith and Larson, '46), reduction in liver catalase and in cytochrome oxidase (Van Reen, '53), depression of heart cytochrome oxidase (Duncan, Gray and Daniel, '53), a decrease in intestinal alkaline phosphatase (Sadasivan, '52) and an increase in liver and kidney alkaline phosphatase (Sadasivan, '52; Van Reen, '53). Since it was found in the above studies that copper supplementation would prevent the anemia and raise the liver catalase and liver and heart cytochrome oxidase to normal or greater than normal values, it has been generally accepted that zinc toxicity precipitates a copper deficiency.

Davis ('58) reported that by increasing the level of zinc in the diet a depression of the copper content in the liver occurs, but only when the copper levels in the diet approach those of borderline or normal of about 5 to 10 ppm. Duncan, Gray and Daniel ('53) reported that the copper content of the hearts of rats was 40% of the normal value when these animals were fed a diet containing 1.0% of zinc and which supplied 0.06 mg of copper per day for a period of 5 weeks. Dick ('54) found that 20 mg of zinc in the daily ration containing 30 mg of copper did not limit copper storage in the liver of sheep, but a significant limitation was found with 100 mg of zinc. Grant-Frost and Underwood ('58) recently reported that feeding a diet containing 0.5% of zinc and 2.1 ppm of copper to growing rats for 6 weeks reduced copper retention. They stated that zinc not only profoundly reduced copper concentration in the blood and tissues but probably also antagonized absorbed copper at the cellular level. They suggested, however, that a deficiency of some other dietary essential affecting hematopoiesis may be occurring as a consequence of the high zinc intake. From work on zinc toxicity of the rice moth, Sivarama Sasty and Sarma ('58) stated that the antagonistic effect of zinc and copper was a reflection of an interference on iron metabolism by zinc which may be a common phenomenon in zinc toxicosis. The present study was carried out to determine what effect a high level of zinc in the diet has on tissue iron and copper that might explain the mechanism of zinc toxicity.

EXPERIMENTAL

Weanling piebald rats of the Long-Evans strain, ranging in age from 21 to 24 days, were used. Males only were used for the time study and an equal number of males and females on each treatment in the other experiments. The rats were individually housed in wire cages with screen bottoms and received feed and distilled water ad libitum. The composition of the basal diet was as follows: casein, 20%; sucrose, 63%; non-nutritive cellulose,[2] 3%; corn oil, 10%; salt mixture, U. S. P. XIV, 4%; 900 I.U. vitamin A, 90 I.U. vitamin D, and 7.5 mg of α-tocopherol per 100 gm of the diet. Other vitamins were provided in the following quantities in milligrams per 100 gm of the diet: thiamine·HCl, 0.5; Ca pantothenate, 5.0; *p*-aminobenzoic acid, 5.0;

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[2] Alphacel.

TABLE 1

Effect of excess zinc and added copper or iron on growth, hemoglobin and hematocrit

Diet	Mean weight gain	Hemoglobin	Hematocrit
	gm	*gm %*	*%*
	Experiment 1 (24)[1]		
Basal	69 ± 6[2]	12.7 ± 6	33 ± 6
0.6% Zn[3]	32 ± 7[4]	8.7 ± 0.5[4]	24 ± 1[5]
	Experiment 2 (40)		
Basal	87 ± 13		
0.5% Zn	59 ± 10[4]		
0.5% Zn + 0.02% Cu[6]	43 ± 7[4]		
0.5% Zn + 0.02% Fe[7]	62 ± 11[4]		
	Experiment 3 (30)		
Basal	165 ± 40	14.5 ± 0.7	39 ± 2
0.4% Zn	129 ± 35[4]	6.9 ± 1.2[4]	20 ± 5[4]
0.4% Zn + 0.01% Cu	126 ± 24[4]	13.0 ± 0.6	38 ± 2
	Experiment 4 (40)		
Basal	165 ± 34	14.9 ± 1.2	38 ± 3
0.4% Zn	129 ± 31[4]	9.1 ± 2.0[4]	28 ± 6[4]
0.4% Zn + 0.04% Fe[8]	122 ± 24[4]	10.7 ± 1.7[4]	31 ± 4[4]
0.4% Zn + 0.08% Fe	140 ± 25[5]	12.2 ± 1.4[5]	33 ± 3[5]

[1] Total number of animals on experiment. Animals were sacrificed after three, 4, and 8 weeks for experiments 1, 2, and 3 and 4, respectively.
[2] Standard error of mean.
[3] Added as zinc oxide.
[4] Significantly less than basal group, $P < 0.01$.
[5] Significantly less than basal group, $P < 0.05$.
[6] Added as $CuSO_4 \cdot 5H_2O$.
[7] Added as soluble ferric phosphate.
[8] Added as $FeSO_4 \cdot 7H_2O$.

riboflavin, 1.0; niacin, 5.0; choline chloride, 100.0; vitamin B_{12}, 0.002; inositol, 10.0; biotin, 0.03; folic acid, 0.05; and menadione, 0.75. The basal and high-zinc diets contained 4 to 5 ppm of copper.

The design of experiments 1, 2, 3 and 4 and the total number of animals on each experiment are shown in table 1. For the time study (table 3), 36 and 18 rats in trials 1 and 2, respectively, were maintained on the following diets: basal and 0.4% of zinc. The supplementary copper or iron was added directly to the dietary mixture. The animals were sacrificed after 3, 4 and 8 weeks on the experimental regimen for experiments 1, 2, and 3 and 4, respectively. In the time study, three rats from each diet were sacrificed after the following periods of time on the diets: weekly for 6 weeks for trial 1, and 1, 3 and 5 days for trial 2. The rats were stunned by a blow on the head and decapitated. With the exception of the animals in experiment 1, the liver and kidneys were perfused *in situ* by inserting a needle into the vena cava and allowing sodium chloride solution (0.9%) to flow through by gravity force until both organs had a pale fawn color. The liver and kidneys were excised, fat and connective tissue removed, rinsed with distilled water and dried to a constant weight at 100°C. The tibia bone was removed, fixed in formalin, decalcified with formic acid and sodium citrate and stained with hematoxylin and eosin.

The method of Peterson and Bollier ('55) was used for the analysis of copper. The zinc content of the tissues was determined by the dithizone method (Shirley et al., '49). The nitroso-R salt method of Sideris ('42) was used for the determination of iron. Hemoglobin level in the blood was measured by the acid–hematin procedure and hematocrit by the conventional method.

The data were analyzed statistically and the multiple range test as proposed

by Duncan ('55) was used to test significance of the means.

RESULTS

Data demonstrating anemia and poor growth caused by three different levels of zinc intake are presented in table 1. In experiment 2, one rat fed the high-zinc diet and one fed the zinc plus iron died in two weeks and are not included in the data. In all experiments, animals fed the high-zinc diets had a poor growth rate ($P < 0.01$), which was not improved by the supplementary copper. Iron supplementation at the 0.08% level appeared to give some improvement of growth; however, this was not statistically significant. The experimental animals fed 0.6 and 0.4% of zinc developed anemia. Rapid blood clotting was experienced with the majority of blood samples obtained from the rats on experiment 2; consequently, insufficient data were obtained to give an accurate picture of the hemoglobin levels. The anemia was characterized by a marked reduction in hemoglobin ($P < 0.01$) and red blood cell volume ($P < 0.05$ for experiment 1 and $P < 0.01$ for experiments 3 and 4). Copper added to the diet containing 0.4% of zinc prevented the anemia. Supplementary iron (0.08%) only partially prevented the anemia.

Rats on the initial experiments did not have a change in the color of haircoat, but those on experiments 3 and 4 developed an achromotrichia. It was characterized by the black hair turning a reddish-brown after three to 4 weeks and gradually to gray. The rats receiving the supplemental copper (experiment 3) did not exhibit achromotrichia, nor was this observed in rats receiving 0.08% of iron.

The data showing the effects of excess dietary zinc and excess zinc supplemented with either iron or copper, on the iron, copper and zinc content of the liver and kidneys and copper in the plasma are presented in table 2. The finding of particular interest in the 4 experiments was the significant ($P < 0.01$) reduction of iron in the liver of rats receiving zinc at a high level. A significant ($P < 0.05$) depression of iron also occurred in the kidneys of rats receiving either 0.5 or 0.6% of zinc. Using liver copper as a criterion, the rats receiving a dietary level of 0.6 or 0.5% of zinc did not show a copper deficiency. However, a marked copper deficiency developed in the rats receiving 0.4% of zinc. This was demonstrated by the lowered ($P < 0.01$) content of copper in the liver and the reduced plasma copper.

The effects of supplementary copper or iron on tissue iron and copper in rats receiving a high level of zinc are of particular interest (table 2). The copper caused a further depression of liver iron. On the other hand, adding iron to the diet of the animals fed the high level of zinc in experiment 2 lessened the severity of the decrease in liver iron and caused kidney iron to be maintained almost at the level found in the rats fed on the basal diet. In experiment 4, the level of iron in the liver of rats receiving 0.08% of iron was essentially the same as for rats fed the basal diet and 0.04% partially overcame the loss. Of particular significance are the data which showed that the added iron (0.08%) prevented completely the loss of liver copper. In experiment 3, the added copper prevented the depression of copper in the liver and plasma.

A marked capacity was found for the rat to absorb zinc and store it in the liver. Neither the copper nor the iron supplementation prevented the buildup of zinc in the liver.

The tibia bone from the rats fed a high level of zinc in experiment 3 showed extensive hyperplasia of the marrow, very few erythrocytes, a considerable increase in the number of myelocytes and megakaryocytes and reticular cell proliferation. In experiment 4, the tibia from rats fed the high-zinc diet showed essentially the same picture. Of interest, however, was the finding that the bone marrow was normal for the rats receiving the high-zinc diets supplemented with either 0.04 or 0.08% of iron.

Since the content of both iron and copper in the liver was reduced in rats receiving 0.4% of zinc for 8 weeks, a time study was designed to determine which element was reduced first. The data ob-

TABLE 2

Effect of excess zinc and added copper or iron on tissue iron, copper and zinc

Diet	Iron[1]		Copper[1]			Zinc[1]	
	Liver	Kidney	Liver	Kidney	Plasma[2]	Liver	Kidney
			Experiment 1 (0.6% Zn)[3]				
Basal (12)[4]	378.4 ± 73.7[5]	229.9 ± 54.6	7.5 ± 1.9	23.8 ± 10.1			
Zn (12)	178.7 ± 51.8[6]	159.3 ± 45.6[6]	8.0 ± 1.8	24.5 ± 9.9			
			Experiment 2 (0.5% Zn)				
Basal (10)	361.9 ± 78.9	105.4 ± 9.5	8.6 ± 1.6	16.9 ± 3.8		290.8 ± 39.2	295.2 ± 83.4
Zn (9)	226.9 ± 60.0[6]	73.0 ± 12.0[7]	9.1 ± 1.7	13.7 ± 3.0		573.7 ± 141.7	801.4 ± 190.4
Zn + Cu (10)	174.6 ± 53.4[6]	95.3 ± 31.0	11.4 ± 3.6	29.2 ± 8.2		421.5 ± 78.7	774.8 ± 157.7
Zn + Fe (9)	269.9 ± 80.0[7]	96.7 ± 25.9	8.1 ± 1.0	11.6 ± 2.4		627.3 ± 83.8	864.4 ± 190.3
			Experiment 3 (0.4% Zn)				
Basal (10)	520.0 ± 186.3		9.8 ± 3.3		81.0	106.7 ± 19.9	
Zn (10)	209.5 ± 40.3[6]		3.9 ± 0.9[6]		21.9	627.6 ± 172.2	
Zn + Cu (10)	144.9 ± 49.8[6]		49.4 ± 19.1		81.0	691.0 ± 234.7	
			Experiment 4 (0.4% Zn)				
Basal (10)	564.0 ± 230.5		8.2 ± 1.8			98.1 ± 9.2	
Zn (10)	123.8 ± 29.9[6]		5.3 ± 1.0[6]			530.4 ± 199.0	
Zn + Fe (10)	340.2 ± 167.6[7]		6.0 ± 1.1[7]			505.6 ± 147.3	
Zn + Fe (10)	486.7 ± 138.9		8.5 ± 2.9			444.1 ± 129.0	

[1] Micrograms per gram of dry weight of tissue.
[2] Micrograms per 100 ml of plasma. Values represent pooled samples.
[3] Animals were sacrificed after three, 4 and 8 weeks for experiments 1, 2, and 3 and 4, respectively.
[4] Figures in parentheses show number of animals assayed.
[5] Standard error of mean.
[6] Significantly less than basal group, $P < 0.01$.
[7] Significantly less than basal group, $P < 0.05$.

TABLE 3

Time effect of excess zinc on liver iron, copper and zinc and hemoglobin and weight gain

Days	Basal[1]					Zinc (0.4%)[1]				
	Mean weight gain	Hemoglobin	Iron[2]	Copper[2]	Zinc[2]	Mean weight gain	Hemoglobin	Iron[2]	Copper[2]	Zinc[2]
	gm	*gm %*				*gm*	*gm %*			
					Trial 1					
7	25	10.7	225.4	12.6	96.9	15	11.0	110.4	11.0	757.7
14	29	12.3	437.6	10.4	105.7	21	10.3	77.6	10.3	515.0
21	24	12.8	449.2	10.0	119.2	14	9.1	61.1	8.0	593.2
28	27	11.6	371.0	10.2	146.5	23	7.5	64.6	6.9	457.6
35	23	14.2	296.5	10.3	136.9	24	8.0	64.5	5.1	560.1
42	17	13.3	335.6	10.6	162.0	4	6.8	72.8	4.5	703.4
					Trial 2					
1	2	10.5	154.8	14.0	135.5	0	11.3	118.5	22.4	349.1
3	13	11.6	154.8	12.8	96.7	7	11.3	78.8	19.3	564.0
5	22	11.6	206.0	13.3	105.3	15	11.1	54.6	14.9	591.6

[1] Values are the average of three rats.
[2] Micrograms per gram of dry weight of tissue.

tained from this study are shown in table 3.

Anemia was evident after 14 days. After 6 weeks the average hemoglobin value of the rats receiving zinc was about one-half that of the controls.

After 7 days on the first trial, the iron content in the liver of rats fed the high-zinc diet had dropped to about 50% of that of the controls. A further loss of iron occurred the second week. Thereafter, the iron remained relatively constant. In the second trial the data show a slight loss of liver iron after one day on the diet and a definite loss after three days. A definite depression of liver copper did not develop until between 21 and 28 days on the high-zinc diet, after which the level continued to drop to the end of the trial.

The buildup of liver zinc in the rats fed the high-zinc diet was very rapid, zinc being absorbed and stored after only one day on the diet. It should be pointed out that the accumulation of zinc in the liver paralleled the loss of liver iron.

DISCUSSION

The finding of particular importance to the understanding of the mechanism of zinc toxicity in the rat was the iron metabolism anomaly, demonstrated by the marked lowering of liver and kidney iron. As shown by the time study, the loss of liver iron was an early manifestation of excess zinc in the diet. The time study also indicated that the loss of iron nearly paralleled the accumulation of zinc in the liver. A reduction of liver copper occurred in rats fed high-zinc diets that did not cause early toxic symptoms but not in those animals receiving 0.6 or 0.5% of zinc for three and 4 weeks, respectively. From the work of Grant-Frost and Underwood ('58), who reported that copper retention was reduced in growing rats receiving 0.5% of zinc for 6 weeks, it is possible that for the present work the rats received the diets of 0.5 or 0.6% zinc for an insufficient length of time. From the time study it was indicated that the loss of liver copper occurred after the loss of liver iron and the development of the anemic condition.

In relation to the production of the anemia, the data indicated that it occurred in rats with a reduced liver iron but with a copper level similar to the controls. Also of significance are the data which demonstrated that additional iron in the diet of rats fed the high-zinc diet apparently prevented the loss of liver copper. From these results it may be stated that (a) excess zinc in the diet causes an early and marked loss of liver iron, (b) the reduction of liver copper occurred after the loss of liver iron and may possibly be the result of the reduction in liver iron,

and (c) zinc toxicity anemia, and presumably the reduced activity of certain iron-containing enzymes, appears to be the result of an iron deficiency rather than copper. The fact that anemia was a relatively late sign of copper deficiency (Gallagher, '57) and that anemia of significant degree was not present in "low copper" rats regardless of an 80% reduction of serum copper and a 40% reduction of liver copper (Dempsey et al., '58) substantiates the present view that zinc toxicity anemia is the result of an iron rather than a copper deficiency.

The action of excess dietary zinc in reducing the amount of iron in the liver is similar to the action of manganese described by Hartman et al., ('55), who noted that feeding high levels of this element decreased the level of iron in the liver of lambs. These workers postulated that manganese interferes with iron absorption rather than with hematopoiesis. Preliminary unpublished work at this laboratory indicated that excess zinc does not interfere with iron absorption. Additional work is needed and is being planned to determine the mechanism as to how excess dietary zinc interferes with the metabolism of iron.

The question arises of why the work of other investigators showed an ameliorating effect of copper on zinc toxicity anemia and decreased activity of some iron-containing enzymes. The results of the present study indicate that the answer lies in the ability of copper to mobilize the iron in the liver even when the iron is at a low level, as demonstrated by the additional loss of iron from the liver of rats fed high-zinc diets supplemented with copper. In this connection, Copp and Greenberg ('46) reported that an intraperitoneal injection of copper to iron-depleted rats fed a diet containing 5.0 ppm of copper resulted in an increased rate of radioactive iron utilization for hemoglobin production. This increased utilization was indicated by a greatly decreased storage of radioactive iron in the liver.

It has been reported that zinc is not normally stored in large amounts in the liver (Underwood, '56) nor absorbed from the intestine in large amounts by the rat (Davis, '58). The results obtained in the present study do not confirm these findings but agree with those obtained with the cat (Scott and Fisher, '38).

As reported by other investigators, symptoms of zinc toxicity exhibited by the rats included poor growth and anemia. The poor growth presumably is caused largely but not entirely by reduced food consumption due to the unpalatability of the diet as suggested by Grant-Frost and Underwood ('53). The increasingly pronounced adverse effect on growth noted in the present study as the zinc content of the diet was increased indicates a corresponding decrease in palatability. The anemic condition was cured by supplementary copper, verifying earlier work. In contrast to the findings of Smith and Larson ('46), the present authors noted that the addition of iron to the high zinc diet appeared to alleviate the anemia somewhat. In the present study, the iron intake was approximately three to 4 times the level used by the above workers, which may account for the difference. In relation to this, whereas the increased amount of bone marrow in the tibia of the rats receiving a high level of zinc was indicative of a compensatory mechanism to alleviate the anemia, the bone marrow was normal in those animals receiving either 0.04 or 0.08% of iron.

Since the achromotrichia occurred in the rats fed the high-zinc diet, with low liver copper, and not in the rats fed the 0.08% of iron in the diet, with corresponding normal liver copper, it is assumed that it was induced by a copper deficiency. The lack of an achromotrichia in the rats receiving either 0.05 or 0.06% of zinc also indicates that they were not copper-deficient. This conclusion stems from the work of Gallagher ('57), who reports that the loss of capacity to produce normally pigmented hair is one of the earliest signs of a copper deficiency.

SUMMARY

A study was made of the effect of excess dietary zinc on iron and copper in tissues of the rat. The results showed that zinc toxicosis results in an accumulation of zinc in the liver with an early and

marked loss of liver iron. The data suggest that the reduction of iron is responsible for the production of the anemic condition and presumably the depression of the activity of some iron-containing enzymes. A lowered liver copper may also occur and the data indicate that it may be the result of the reduced liver iron rather than an effect of the zinc. Copper probably acts in counteracting the anemia and reduced enzyme activity of zinc toxicity by further mobilizing the iron in the liver.

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Nutritional Properties of Fresh Fats Added to Diets Containing Autoxidized Cottonseed Oil[1]

HANS KAUNITZ, C. A. SLANETZ, R. E. JOHNSON AND VIGEN K. BABAYAN

Department of Pathology and Institute of Comparative Medicine, Columbia University, New York, and Research and Development Laboratories, E. F. Drew and Company, Inc., Boonton, New Jersey

Diets containing highly autoxidized cottonseed oil lead to rapid weight loss when fed to rats and result in a high death rate. Addition of fresh cottonseed oil has been observed to exert a protective effect (Kaunitz et al., '55). In these studies, it was not clear whether this protective effect was a property of any fresh fat or whether various fats differ in this respect. This question invited further studies, particularly because it had meanwhile been observed that medium-chain and long-chain saturated triglycerides frequently differ in their nutritional effects (Kaunitz, et al., '58a, b; '59). Furthermore, such studies could be helped by the more detailed information which had been obtained as to the effect of such oxidized fatty materials on water intake and organ weights (Kaunitz, et al., '56, '59, '60).

MATERIALS AND METHODS

Refined cottonseed oil was aerated at 95°C for 300 hours. This oxidized cottonseed oil (OCSO) was included at levels of 10 and 15% in a purified diet shown in table 1. When desired, 20 or 15%, respectively, of a fresh fat was added to the diet at the expense of the carbohydrate. All diets were kept refrigerated.

The fresh fats used were commercially-available, refined cottonseed oil (CSO), refined corn oil, refined coconut oil, sweet butter, refined olive oil, and soybean oil and freshly rendered leaf lard, chicken fat, and perirenal beef tallow. In addition, medium-chain and long-chain saturated triglycerides (MCT and LCT) and ethyl esters of CSO were studied. The MCT was prepared from coconut oil by fractionation of the split fatty acids and reconstitution of the desired fraction (6 to 12 C) into triglycerides. The oil was clear, thin, odorless, with a melting point below 0°C and an iodine value of less than one. LCT was derived from coconut or other palm-kernel oils by hydrogenation of the fatty acids of 14 to 18°C and their reconstitution into triglycerides. This material had a melting point of about 40°C and an iodine value of 3 to 5. The ethyl esters of CSO were prepared by refluxing the oil with aqueous NaOH in alcohol, acidification and esterification with ethanol.[2]

The weanling rats used were males (except for one series) derived from a colony of the Sherman strain. When they were delivered to the laboratory at 24 days of age, they were placed on a diet similar to that in table 1 but containing lactalbumin instead of casein and 10% of fresh lard as fat. In about 5 days they were earmarked and weighed. After reweighing 4 or 5 days later, they were distributed into matching groups of 8 rats (7 in some series) so that average body weights were the same for the first weighing and again for the second weighing. The rats were placed in individual cages and supplied with non-dripping water bottles suitable for water consumption measurements. Vitamin supplements were fed by dropper to compensate for destruction of vitamins in the diets containing oxidized fats. The rats were weighed at least once weekly. After three weeks (4 in some instances), they were

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[2] Dr. Daniel Swern and Mr. H. B. Knight of the Eastern Regional Research Laboratories of the U. S. Department of Agriculture suggested the use of the esters and prepared them for us.

TABLE 1
Composition of purified diet containing oxidized cottonseed oil

Ingredient	Amount
	%
Oxidized cottonseed oil	10 or 15
Dextrose[1]	54 or 49[2]
Casein[3]	30
Cellulose[4]	2
Salt mixture (USP XIII)	3.5
Calcium carbonate	0.5
Vitamins and accessory factors[5,6]	

[1] Cerelose.
[2] When fresh fat was fed with the oxidized cottonseed oil, it was added at the expense of the carbohydrate.
[3] G.B.I. Vitamin-Free.
[4] Alphacel.
[5] For details, see J. Nutrition, 64: 514, '58.
[6] We wish to thank Dr. Leo A. Pirk of Hoffman-La Roche, Inc., Nutley, New Jersey, for most of the synthetic vitamins.

anesthetized with chloroform, as much blood as possible was withdrawn from the heart and the organs were weighed. The ventricles, rather than the whole heart, were weighed because they can be separated with considerable accuracy.

Organ weight data are presented in figure 1 as a log-log plot of organ weight against body weight. This method was used because the ratio of the weight of an organ to the corresponding body weight is not linear but varies continuously, and a log-log plot gives a straight line distribution for most organs which may have one or more changes in slope with increasing body weight. The distribution has a uniform spread throughout its range. The normal organ weight distributions used for comparison in these experiments were derived from organ weight data collected for over 4 years from 427 male rats fed a diet similar to that given in table 1 but with fresh lard as fat source. The weights of an organ were grouped according to the corresponding body weights so that the body weight range for each subgroup was small. The organ weights of each subgroup were averaged and this average was plotted against the body weight representing the midpoint of the group range. A straight line resulted which had the same slope as the distribution. This line became the source of the "normal" weight of the organ for any given body weight. These lines are given in figure 1.

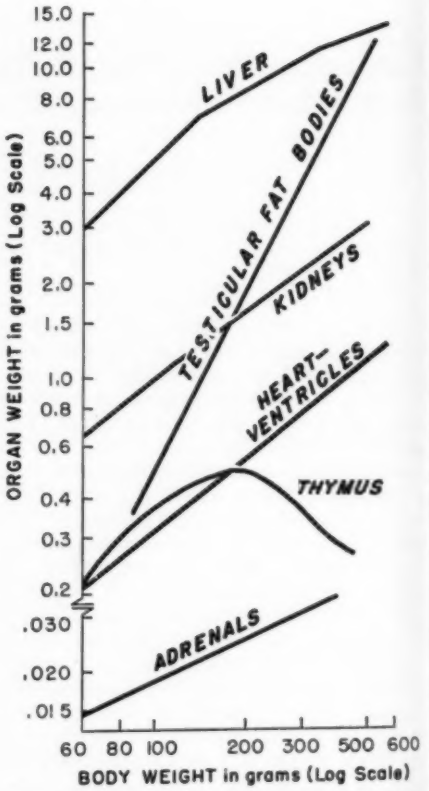

Fig. 1 Lines derived from plotting average organ weight-body weight relationships on a log-log scale.

The actual weight of an organ of an experimental animal was compared with the "normal" weight of the organ for the animal's body weight as derived from the appropriate line, and the difference between the two was expressed as a percentage of the normal weight. The individual percentages were used for statistical analyses. Although this method avoids some difficulties in comparing weights of animals of different body weights, it may have its limitations. One can hypothesize that a particular treatment could lead to the maximum enlargement of a certain organ for animals of a given age, which would make any differences in percentages of enlargement specious; the latter would

represent only differences in body weight rather than true differences in effect on the organ.

For statistical purposes, P values were calculated by t tests. A P value of 0.05 was considered as just significant. The ± values given are standard errors.[3]

EXPERIMENTS

Ten experimental series were carried out in which OCSO was fed alone and with various fresh fats. In order to facilitate the presentation of the data, all results involving any one fresh fat were combined and compared with the data from the corresponding groups fed OCSO alone.

In figure 2 are given the survival rates and the differences in body weight between corresponding series on OCSO alone and with an added fresh fat. When only 10% of OCSO was fed, most animals survived. With 15% of OCSO alone, a considerable number of animals died; with 15% of MCT, CSO and chicken fat, the survival rates were higher. The chi square for OCSO + MCT compared with OCSO alone was 5.8 and for chicken fat, 4.6.

When OCSO alone was fed, most rats lost weight. The groups fed 10% lost an average of 6 gm and those fed 15%, 16 gm (not significant). There was a decided difference in how the addition of fresh fats influenced the body weights. The use of 20% added to 10% of OCSO, MCT and CSO led to body weights which were 33 and 41 gm higher, respectively, than their controls ($P < 0.001$ for each). With 20% of lard and 20% of ethyl esters of CSO, the differences were less pronounced but still significant; with LCT, average body weights were lower than those with OCSO alone, but not significantly.

The use of 15% each of OCSO and fresh fat, MCT, corn oil, olive oil, CSO, and soybean oil very significantly prevented body weight losses; the action of lard was somewhat less pronounced. With chicken fat, the difference in body weight over the OCSO controls was not quite on the borderline of significance. Butter, coconut oil, and the ethyl esters of CSO had no effect, and beef significantly aggravated the condition.

Water intake measurements were carried out in three series. For purposes of comparing the intakes of animals of widely

[3] Dr. John W. Fertig of the Department of Public Health and Administrative Medicine, Columbia University, kindly helped us with the statistical analyses.

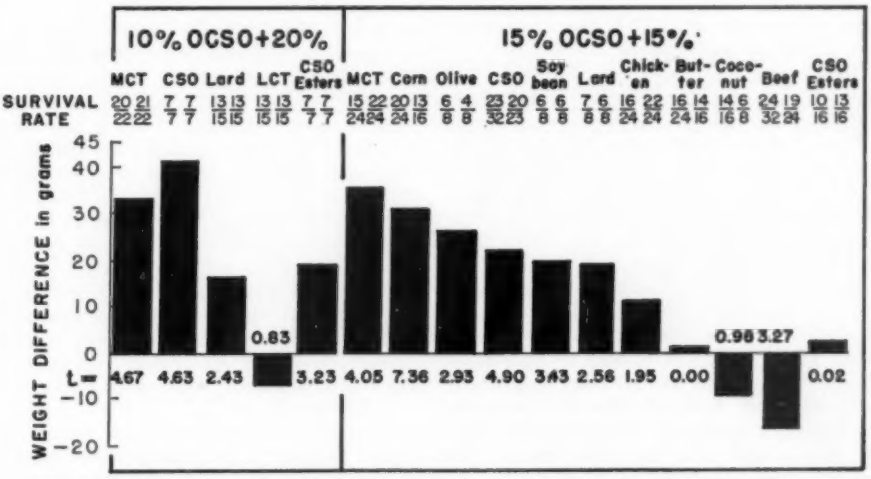

Fig. 2 Influence on body weight and survival rate of fresh fat added to a diet containing OCSO. Effects on body weight are expressed as weight difference between corresponding groups on OCSO alone and with a fresh fat. The t values refer to these differences. In each pair of survival rates, the first refers to that with OCSO alone.

differing body weights, intakes were expressed in terms of body surface. The body weights for each animal for the period of measurement were averaged and average surface calculated according to Lee's formula, $S = 12.54\ W^{2/3}$ where S is the surface in cm^2 and W is the weight in grams (Lee, '29). The average weekly water intake of each rat for the three-week period was divided by its S/100 to give $cm^3/100\ cm^2/week$. Five control groups fed only fresh fat (not otherwise used in these studies) had average intakes of 36 to 38 $cm^3/100\ cm^2/week$ for body weights of 100 to 428 gm. The groups fed OCSO alone had intakes of $66 \pm 8.6\ cm^3$ with 10% and, in the two series fed 15%, 85 ± 3.0 and $7.3\ cm^3$. This is in agreement with the finding that some fractions of such oxidized oils greatly increase water intake (Kaunitz et al., '59). Rats fed OCSO and 20% of MCT had an intake of $48 \pm 3.6\ cm^3$; with lard, it was $55 \pm 4.1\ cm^3$ and with LCT, $72 \pm 9.4\ cm^3$. The difference between the intakes of those fed OCSO alone and those fed OCSO with MCT were just significant; between those fed MCT and LCT, it was more pronounced. With 15% each of oxidized and fresh fats, MCT and other fats had no influence. Therefore, MCT was capable of reducing the high water intake associated with the intake of OCSO, but only when fed at rather high levels.

In figure 3 are presented the more pertinent data on the organ weight-body weight relationship. Average organ weight values and standard errors are also given. Kidney values show that the kidney was enlarged using both levels of OCSO alone; MCT and CSO led to significantly less enlargement when 20% was fed. With 20% of LCT, the percentage of deviation from normal was higher than with OCSO alone, but not significantly so. Lard and coconut oil had no effect even at the 20% level. With 15%, only MCT had a significant effect; beef fat aggravated the condition. CSO, corn oil, lard, chicken fat, coconut oil, butter, and the ethyl esters of CSO had no effect and are not included in the figure.

The enlargement of adrenals ran more or less parallel with that of the kidneys. OCSO alone led to adrenals with an average weight which was higher than their calculated weights at the start of the experiments as derived from the adrenal weight-body weight line. This increase occurred while the animals lost weight. With both levels of MCT, the percentage of deviation was less. With 20% of LCT, the adrenals were significantly heavier than in the corresponding groups on MCT although the body weight of the latter groups was so much higher.

Lard and coconut oil fed at the 20% level and CSO, chicken fat, lard, butter, beef fat, and the ethyl esters of CSO fed at the 15% level were studied; they had no influence and are not given in the tables.

The degree of liver enlargemnt was significantly reduced by 20% MCT and CSO but not by 20% of LCT. Also studied, but not included in the table, were lard at the 20% level and MCT, CSO, corn oil, chicken fat, lard, coconut oil, butter, beef fat, and ethyl esters of CSO, none of which had any effect.

Thymus weights were reduced more than 50% when OCSO alone was fed. MCT and fresh CSO reduced the losses significantly; ethyl esters of CSO did not. Also studied, but not included in the table, were lard on the 20% level and lard, butter, and beef fat on the 15% level. They had no effect.

Testicular fat bodies were weighed because it has been shown that the weight of these is proportional to the total neutral fat in the rat (Hausberger, '37; Stoerk and Porter, '50). With OCSO alone, the weight of this organ was consistently below normal. All fresh fats except beef fat and the ethyl esters of CSO increased the weight of the testicular fat body in relation to the body weight, i.e., increased the total neutral fat depots. MCT, although counteracting body weight losses at least as well as fresh CSO, led to smaller fat bodies than did CSO. This is in agreement with the observation that MCT does not easily induce deposition of neutral fat (Weitzel, et al., '55; Kaunitz et al., '58b).

Changes in the heart ventricle weight-body weight relationship were not pronounced and are not included in the figure. However, one must take into consideration that the standard deviation of ventricle weights is the smallest one for any

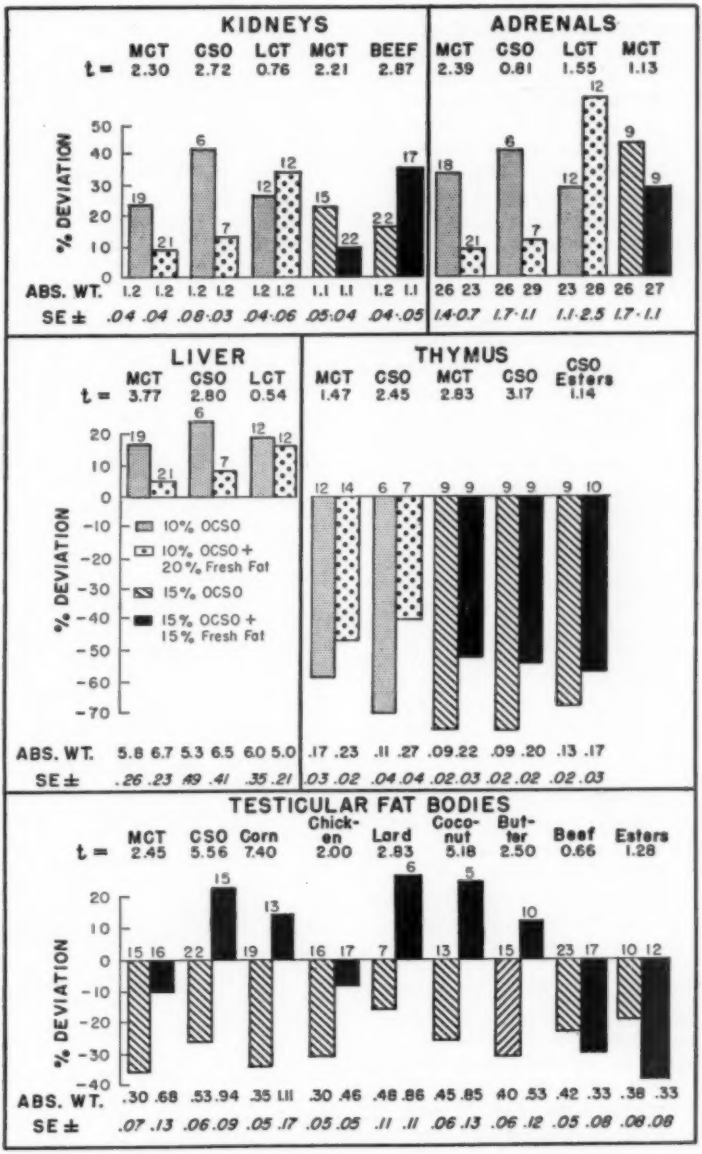

Fig. 3 Influence on organ weight-body weight relationship of adding fresh fat to a diet containing oxidized cottonseed oil (OCSO). Effects are expressed as percentages of deviation from normal. The t values refer to differences in percentage of deviation between corresponding groups fed OCSO alone and with a fresh fat. Numbers at the top of the columns are number of observations; below are given the average absolute organ weights in grams (milligrams for adrenal weights).

organ. It was noted in all series that OCSO alone induced ventricular weights approximately 5% smaller than expected from the standard line. This was also true when MCT, CSO, chicken fat, lard, coconut oil, and the ethyl esters of CSO were fed. With LCT and beef fat, the weights were about 2% above normal. When the data from corresponding series fed MCT and LCT were compared statistically, a P of 0.02 resulted; thus, ventricle weights were relatively higher when LCT was fed.

From some of the aforementioned observations, it was evident that the ethyl esters of CSO gave much less protection against weight losses and organ changes than did fresh CSO. In order to establish whether the glycerol moiety had any effect, one group was given 5% of glycerol with 10% of OCSO. Neither body weights nor organ weights were different from those of the group fed OCSO alone.

DISCUSSION

The various fats can be divided, with respect to their effect on body weights, into three groups: those strongly counteracting weight loss (MCT, CSO, corn oil, olive oil, and soybean oil), those having little or no influence (lard, coconut oil, butter, and chicken fat) and those aggravating the condition (LCT and beef fat).

Comparison of effects on organ weights of the fats strongly counteracting body weight losses shows that there are differences. Only MCT exerted a beneficial effect on kidneys and adrenals, and it also induced less neutral fat deposition than the other fats. Thus, a certain specificity in the action of these fats can be assumed.

If one attempts to relate any of the biological effects of these fresh fats to their physical properties, considerable correlation seems to exist between melting point and effect on body weight. Those with a low melting point (MCT, corn oil, CSO, soybean oil, and olive oil) gave the most protection against weight loss. Those with high melting points (beef fat and LCT) increased weight losses. Lard, chicken fat, butter, and coconut oil formed an intermediate group with respect to melting point and effect.

That the beneficial effect depends upon the presence of the fatty acids as triglycerides is suggested by the fact that the ethyl esters derived from CSO had little effect and neither did glycerol. Furthermore, although fats containing high percentages of linoleate in triglycerides (corn oil) had a beneficial effect, the addition of ethyl linoleate, in one experiment, to a diet containing OCSO led to the death of all 8 animals within 10 days. Those fed ethyl linoleate alone were normal.

The beneficial effect of certain oils is, in some ways, paradoxical. Previous studies (Kaunitz et al., '55) have shown that addition of fresh CSO to a diet containing OCSO led to increased food consumption and, therefore, to increased intake of the toxic material itself. Moreover, paired feeding experiments have shown that animals given fresh CSO in addition to OCSO did not show the same weight loss or organ enlargement; this is evidence that the findings are not the result of hunger, per se. Of some relevance may be the observation that polymerized fats decrease lipase activity in the feces (Peretti and Reale, '36) and reduce fat absorption[4] (Lassen et al., '49). If it is possible that the melting point of a fat influences both enzyme activity and food absorption, the difference between protective and non-protective fats may rest partly in their intestinal activity. However, this would not explain why fats with low melting points, which increase the absorption of oxidized material, have a beneficial effect. One may speculate that there exists competitive antagonism between oxidized and fresh fats after absorption.

The data may have some relation to the question of why fats have beneficial effects in some stress conditions. Feeding of OCSO subjects the animal to severe stress, and it may be of general interest that this stress is counteracted by liquid fats and aggravated by hard fats. The saturated but liquid fat, MCT, had effects

[4] Saunders, D. H., H. B. Knight, D. Swern, H. Kaunitz, C. A. Slanetz and R. E. Johnson 1957 Composition of fecal lipids of rats fed diets containing polymers from autoxidized fats. Abstracts of the 48th Annual Meeting, Am. Oil Chemists' Society, no. 48.

at least as beneficial as the highly unsaturated oils.

SUMMARY

1. Purified diets containing 20 or 15% of medium-chain saturated triglycerides (MCT), refined cottonseed oil (CSO), corn oil, chicken fat, lard, coconut oil, butter, beef fat, long-chain saturated triglycerides (LCT) and ethyl esters derived from CSO in addition to 10 or 15%, respectively, of cottonseed oil aerated at 95°C for 300 hours (OCSO) were fed to weanling rats for three weeks.

2. Survival rate was significantly improved by MCT.

3. Body weight was considerably increased by MCT, CSO, corn oil, olive oil, and soybean oil; mildly improved by lard and chicken fat; not influenced by butter and coconut oil; and lessened by beef fat and LCT.

4. The elevated water intake of animals fed OCSO was reduced by MCT when 20% was added to 10% of OCSO.

5. Studies of organ weights in relation to body weight showed that the enlargement of kidneys and adrenals produced by OCSO was significantly reduced by MCT and fresh CSO, accentuated by beef fat and LCT and unaffected by other fats. Heart ventricle weights, in comparison with those of normal animals, were somewhat reduced with OCSO alone and with MCT and mildly increased with beef fat and LCT. (The difference between LCT and MCT was significant). The liver enlargement observed with OCSO was reduced by 20% of MCT or CSO. The reduction of testicular fat body weight associated with the feeding of OCSO was counteracted by most fresh fats.

6. The beneficial effect of the ethyl esters of CSO was slight compared with that of fresh CSO.

7. It is suggested that the beneficial effect of triglycerides on body weight can be correlated with their melting point.

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The Effect of Limited Water Intake on Nutrient Utilization[1]

FRANK KONISHI[2] AND C. M. McCAY
Animal Nutrition Laboratory, Cornell University, Ithaca, New York

It has been suggested that a regulatory mechanism exists whereby water intake is quantitatively related to food intake (Strominger, '47). Studies with mice (Bing and Mendel, '31), rats (Jackson and Smith, '31; Adolph, '47; Strominger, '47; Crampton and Lloyd, '54; Lepkovsky et al., '57), poultry (Wilson and Edwards, '52; Slinger and Pepper, '55) and cattle (Larsen et al., '17; Balch et al., '53) have shown that restriction of either food or water results in a decreased intake of the other. In most of the studies cited, water was the limiting factor involved which resulted in a lowering of food consumption and consequent inhibition of body weight gain and growth.

Adequate studies of the relation of water intake to level of food consumption are thus available. However, there is a paucity of experimental data delineating the effect of water limitation upon food utilization. In an extensive study with ruminants, Larsen and co-workers ('17) found an increase in the apparent absorbability of various nutrients when the interval between watering was lengthened. This increase was most apparent with crude fiber. Balch et al. ('53) also noted slight increases in absorption of crude fiber by cows when water was limited.

The present study was designed to investigate further the effect of water restriction on nutrient utilization as determined by balance studies with dogs consuming diets relatively high in either protein, fat or carbohydrate.

EXPERIMENTAL

Six adult male Beagle dogs, averaging 9.1 kg in body weight and 21.7 months in age were used in this study. Each dog was given a constant amount of food and of such quantity so that body weight was at least maintained. The animals were housed and fed in individual metabolism cages throughout the study. The three diets which were designated as either high protein, fat or carbohydrate, consisted of a basal mix in percentage of corn flakes, 10; wheat germ, 4; boneles meat meal, 5; tomato pomace, 3; brewers' yeast, 3; irradiated yeast, 1; cod liver oil, 1; calcium-free salt mix, 2.6; and $CaHPO_4$, 0.4. The remainder of the three diets was supplied by sucrose, casein and lard as follows (per cent): high protein diet, sucrose, 45; casein, 20; and lard, 5; high fat diet, sucrose, 45; casein, 5; and lard, 20; and high carbohydrate diet, sucrose, 50; casein, 10; and lard, 10.

The plan of the experiment was to allow water ad libitum during a control balance period while consuming a given diet. This was followed by another balance period during which each dog ate the same diet but was limited in its intake of water to approximately one third of the control intake. The dogs were assigned randomly to the three diets during a given test period and each dog was exposed eventually to each of the three diets and treatments. A constant quantity of water was mixed with a particular diet during both periods. Water during the restricted period was usually offered 10 to 15 minutes post prandially in regular stainless steel feeding dishes. Corrections were not made for loss of drinking water by evaporation. The temperature of the metabolism room varied from 14.4°C to 16.1°C during the 60-day

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[2] Data taken from a part of a Ph.D thesis submitted to the Faculty of the Graduate School of Cornell University. Present address: U. S. Naval Radiological Defense Laboratory, San Francisco 24, Calif.

test period. No attempt was made to maintain the relative humidity of the room constant.

Each of the 6 balance studies consisted of a 6-day preliminary period followed by a 4-day collection period. Chromic oxide was added to the diet as an indicator each morning to the extent of one per cent of the total dry matter. Total daily urines were obtained and a 20% aliquot retained and pooled for the 4 days. Representative fecal samples were collected daily, dried overnight at 80°C and pooled for the 4 days. Protein was determined by the Kjeldahl technique, and moisture, ash and ether extract by regular A.O.A.C. methods ('50). Total carbohydrate was obtained by difference. Calcium was precipitated as the oxalate and titrated with 0.05N $KMnO_4$. Phosphorus was determined according to the colorimetric method described by Simonsen et al. ('46). Chromic oxides were measured by the sodium peroxide fusion method described by Schürch et al. ('50).

RESULTS

Limiting the intake of drinking water did not affect appetite as evidenced by the immediate consumption of the diets by the dogs. The animals appeared to be in good health and, in 15 out of 18 observations, there was maintenance or gain of body weight during the restricted water period. The feces seemed to have a lower water content during the restriction period but daily defecations occurred throughout the test periods.

The pattern of water consumption during the restricted water periods varied between individual dogs. Some dogs immediately drank the total water offered whereas others would drink only a portion at the first draft. The average water consumption during the control periods was essentially the same irrespective of diet (table 1). Urine volumes were, of course, markedly reduced during the limited water period, but the differences in volume between diets were not significant.

The average volume of urine excreted per volume of water ingested during the control period was remarkably constant on the three diets. The ratios also were constant between diets but significantly lower ($P < 0.01$) during the limited water periods, namely, the urine was more concentrated.

The maintenance of body weight during the restriction period was unexpected. More surprising was the apparently greater increment of gain during the test period as compared with the control using the fat and carbohydrate diets.

Restriction of water while consuming the three diets did not influence the apparent absorption of calcium as shown in table 2. However, the excretion of calcium by the kidneys was significantly lower during water limitation ($P < 0.05$), the excretion being lowest on the fat diet. The retention of calcium was inversely related to urinary excretion, being highest during water limitation and also highest on the fat diet.

TABLE 1

Mean water intakes and retentions, urine volumes, urine-water ratios and body weight changes in 6 dogs during control and limited-water intake periods, using three diets

	Diet					
	High protein		High fat		High CHO	
	Control[1]	Limited water[2]	Control	Limited water	Control	Limited water
Water intake, ml/day						
Feed	150	150	65	65	130	130
Drinking	811	275	930	308	942	317
Total	961	425	995	373	1072	447
Urine volume, ml/day	644	201	683	176	711	218
Water retained, ml/day	317	224	312	197	361	229
Urine-water intake ratio	0.67	0.47	0.69	0.47	0.66	0.49
Body weight change, gm/day	+28.9	+9.4	+9.9	+17.1	+17.5	+24.1

[1] Control, water allowed ad libitum.
[2] Limited water, approximately one-third of control intake.

TABLE 2

Mean intake, apparent absorptions, urinary excretion and retentions of calcium, phosphorus, and nitrogen during control and limited water intake periods, using three diets

Diet	Period	Intake	Apparent absorption	Urine	Retention
		gm/day	%	*gm/day*	%
			Calcium[1]		
High protein	Control[2]	0.493	50 ± 4.1[3]	0.169 ± 0.013	32 ± 2.7
High protein	Limited[4]	0.493	47 ± 4.3	0.135 ± 0.017	42 ± 5.2
High fat	Control	0.416	49 ± 3.8	0.114 ± 0.009	43 ± 3.3
High fat	Limited	0.416	53 ± 2.5	0.103 ± 0.011	54 ± 3.0
High CHO	Control	0.423	53 ± 1.3	0.150 ± 0.013	34 ± 4.0
High CHO	Limited	0.446	56 ± 4.0	0.139 ± 0.014	45 ± 3.5
			Phosphorus[5]		
High protein	Control	0.897	77 ± 1.4	0.522 ± 0.024	25 ± 2.3
High protein	Limited	0.897	76 ± 1.4	0.245 ± 0.026	64 ± 3.8
High fat	Control	0.655	78 ± 2.6	0.377 ± 0.014	26 ± 1.5
High fat	Limited	0.655	78 ± 2.7	0.215 ± 0.022	57 ± 5.4
High CHO	Control	0.728	75 ± 0.2	0.397 ± 0.020	28 ± 3.1
High CHO	Limited	0.754	77 ± 1.2	0.265 ± 0.024	54 ± 6.0
			Nitrogen[6]		*gm/day*
High protein	Control	10.96	93 ± 0.5	6.66 ± 0.37	3.51 ± 0.15
High protein	Limited	10.85	94 ± 0.4	6.13 ± 0.29	4.02 ± 0.22
High fat	Control	4.25	83 ± 1.1	3.42 ± 0.14	0.34 ± 0.20
High fat	Limited	4.25	84 ± 1.2	2.75 ± 0.08	0.91 ± 0.13
High CHO	Control	5.74	87 ± 0.7	4.07 ± 0.16	0.93 ± 0.14
High CHO	Limited	5.99	89 ± 0.6	3.88 ± 0.20	1.44 ± 0.11

[1] Urinary excretions significantly lower ($P < 0.01$) and retentions significantly higher ($P < 0.01$) during limited water intake periods.
[2] Water allowed ad libitum.
[3] Standard error.
[4] Water approximately one third of control intake.
[5] Urinary excretions significantly lower ($P < 0.01$) and retentions significantly higher ($P < 0.01$) during limited water intake periods.
[6] Apparent absorptions higher ($P < 0.01$), urinary excretions lower ($P < 0.01$), and retentions higher ($P < 0.05$) during limited water intake periods.

As with calcium, phosphorus absorption was not influenced by restriction of water or by diet (table 2) and similarly, the urinary excretion of phosphorus was significantly lower ($P < 0.01$) and the retentions were significantly higher ($P < 0.01$) when water was limited. Since the total quantity of phosphorus absorbed was greatest with the protein diet, the urinary excretion was significantly higher ($P < 0.01$) than with either the fat or carbohydrate diet. As a consequence, the percentage of retentions was quite similar between diets.

Unlike calcium and phosphorus, the apparent absorption of nitrogen was significantly higher ($P < 0.01$) when water was limited. The magnitude of the mean differences was not great but the individual values were consistent in the same direction. The retentions of nitrogen expressed as grams per day were also significantly higher during water limitation. A consistent and significantly lower ($P < 0.01$) urinary nitrogen was also observed during the test period. Differences in utilization of the nitrogen between diets were all significant at the 1% level, reflecting differences of dietary levels.

The mean apparent absorptions of dry matter, ether extract and carbohydrate are shown in table 3. The differences in the apparent absorptions of dry matter and total carbohydrate were significant upon statistical analysis, but from a practical viewpoint, the differences are so minor that they may be considered essentially the same between the control and limited water periods. Dry matter was uniformly utilized between the three diets. The absorption of ether extract was unaffected by water intake, however the utilization

TABLE 3

Mean apparent absorptions of dry matter, ether extract and carbohydrate during control and limited water intake periods, using three diets

Diet	Period	Apparent absorption		
		Dry matter	Ether extract	Carbohydrate
		%	%	%
High protein	Control	91.2 ± 0.5[1]	92.5 ± 0.5	92.3 ± 0.4
High protein	Limited	91.8 ± 0.4	93.2 ± 0.5	92.8 ± 0.4
High fat	Control	90.9 ± 0.6	95.6 ± 1.3	92.9 ± 0.5
High fat	Limited	91.8 ± 0.3	95.9 ± 0.4	93.7 ± 0.3
High CHO	Control	91.5 ± 0.2	94.8 ± 0.4	94.0 ± 0.3
High CHO	Limited	92.0 ± 0.3	95.0 ± 0.6	94.2 ± 0.3

[1] Standard error.

was significantly lower ($P < 0.01$) with the protein diet than with either the fat or carbohydrate diet.

DISCUSSION

Maintenance or increases in body weight during water restriction were not observed by other investigators (Jackson and Smith, '31; Adolph, '47; Crampton and Lloyd, '54; Slinger and Pepper, '55) in studies with young growing animals. It is possible that mature animals, as in this study, have a lower water requirement for maintenance. The findings may also reflect overcompensation of urine concentration by the kidneys when the water was restricted. It is recognized, however, that the weight data in the present study represent changes over a period of only 10 days' restriction.

Schreiber and Elvehjem ('55) found that rats fed high-fat or high-protein diets with limited water intakes lost more weight than those fed high carbohydrate diets. They attributed these results in part to the little-recognized fact that fat and protein contribute less metabolic water per calorie than carbohydrate. This fact may explain also the apparent greater increase in body weight observed in 5 of the 6 dogs during the limited water period when fed the high carbohydrate diet in this study.

The absorptions of calcium and phosphorus were not influenced by limitation of the drinking water, possibly since the water added to the diet was the same during both control and test periods, plus the availability of water immediately after eating. Indeed, Lepkovsky and co-workers ('57) found that the gastric contents of rats fed with or without water were always approximately 49% of water. The additional water was subsequently found to be furnished by the skin, adipose tissue and possibly other tissues as shown by moisture analysis of the various tissues. Should this mechanism occur in the dog as well as in the rat, it would tend to maintain normal gastric flow necessary for adequate assimilation of the above minerals.

The decrease in urinary excretion of calcium and phosphorus during the water restriction period is probably a reflection of available water necessary for maximal concentration and solution of the minerals. Whether this decrease would be desirable or undesirable in situations of dietary deficit is still unknown. However, the relationship of abnormal mineral metabolism to renal disease, particularly renal calculi, certainly warrants additional study.

An increase in apparent nitrogen absortpion was also obtained by Larsen and co-workers ('17) when water was limited. The decrease in urinary nitrogen during the test period was observed also by Larsen's group but not by Black et al. ('44) and Grande et al. ('57). The latter investigators found increased urinary nitrogen which they considered as a metabolic response to the stress of dehydration. Grande et al. ('57) found that even limiting the water intake in a group of healthy men produced a greater urine volume than in other groups of men on a more liberal water intake. They were, however, subsisting on a low calorie diet devoid of protein.

The slight increase in apparent absorption of dry matter and carbohydrate during water limitation is similar to the earlier findings of Larsen et al. ('17) with rum-

inants. Balch et al. ('53) also noted small increases in absorbability of dry matter due to an increase in crude fiber utilization, and of the total digestible energy of hay in dairy cattle. Larsen et al. ('17) suggested on the basis of his studies that water be withheld just before or after a heavy meal for the most efficient digestion of the feed. On the other hand, Lepkovsky and co-workers ('57) observed similar rates of absorption regardless of the availability of water during eating.

An explanation for the apparent increase in absorption of nitrogen, carbohydrate and total dry matter when water is limited is unknown. It is not unreasonable to suppose that under conditions of water restriction, gastric and intestinal secretions as well as motility may be modified in some manner so that absorption is facilitated. It is recognized, however, that this study was of short duration and may not depict the normal or actual consequence of limited water supplies under natural conditions. It is of interest to note that the results of this study were similar to those obtained with ruminants which depend to a large extent upon bacterial action in the rumen for adequate utilization of the food.

SUMMARY

Balance studies were conducted using adult male dogs to investigate the influence of limiting the intake of water upon nutrient utilization. Limitation of the drinking water to approximately one third of that consumed during a control ad libitum period resulted in a marked reduction in urine volume but little change in body weight while consuming diets relatively high in either protein, fat or carbohydrate.

The apparent absorptions of calcium and phosphorus were unaffected by water restriction but urinary excretions were significantly lowered, whereas the retentions were increased. The utilization of nitrogen was significantly greater during the test period along with decreased urinary losses. The apparent absorptions of ether extract, dry matter and carbohydrate were essentially unaffected by water restriction although with the latter two nutrients, the slight differences in utilization obtained were statistically significant.

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Dental Caries and Growth in Rats Fed Whole-Milk Powders with Increasing Lysine Deterioration

H. D. CREMER, H. J. KINKEL AND J. MAURON
Institut für Ernährungswissenschaft der Justus Liebig-Universität, Giessen, Germany, and Research Laboratories of Nestlé's Products, Vevey, Switzerland

From several caries experiments using rats fed heat-processed proteins, McClure and Folk ('55b) concluded that a destruction of lysine or a modification brought about in the lysine molecule by the heat treatments are responsible for the cariogenic effect. They had shown ('53) that a diet[1] containing 35% of dry skim milk powder, autoclaved 15 minutes at 121°C, was highly cariogenic in rats. There was no direct relationship between growth impairment and caries incidence.

With both strains of rats used in these experiments, i.e., Sprague-Dawley and Holtzman, caries of the smooth-surface type was observed in 84% of the animals. With the same diet, rats of the Osborne-Mendel strain exhibited nearly as much caries of the smooth surface as of the occlusal fissure type (Losee and Nemes, '54). Whether caries of the occlusal fissure type, which we also observed in our rats of the Osborne-Mendel and Wistar strains, or caries of the smooth-surface type appear, seems therefore not to be a matter of diet, but rather one of animal strain; we may assume that it is primarily due to genetic predisposition.

In another investigation, McClure and Folk ('55a) found that the incidence of caries was greater the more drastic the heat treatment during milk drying. With lyophilized skim-milk powder, caries incidence was lowest.

In earlier experiments, McClure ('52) obtained an increased incidence of caries with a diet containing 25% of both dried-rye and wheat bread, enriched with vitamins and salts; 15% of both cooked-oat and corn flakes, which had been subsequently dried; 18% of glucose; 2% of NaCl, plus adequate amounts of vitamins. Growth was not optimal with this diet.

In more recent experiments, McClure ('58) again observed an increased incidence of caries by feeding autoclaved wheat flour. Toasted wheat bread showed, however, no higher incidence of caries. Here again a relationship between growth impairment due to heat treatment and caries severity could not be demonstrated.

It is the purpose of the present investigation to examine whether lysine deficiency or some unknown factor produced by the heat treatment of milk caused the increased caries frequency in our strain of rats as shown by the "short term technique" (Mühlemann and others, '56).

EXPERIMENTAL

The whole-milk powders were prepared in Nestlé's Orbe plant (Switzerland) by the continuous spray process and by drying on twin-cylinder atmospheric rolls. Fresh milk was pasteurized for a short time at approximately 110°C, concentrated in the vacuum pan at 48°C to a total solid content of 35 to 37%, homogenized, cooled to 12°C and dried. In addition to the ordinary roller-dried milk which was subjected to steam pressure of 6.5 atmospheres at a speed of 11 rpm, slightly-scorched roller powders with increasing lysine deterioration were prepared by progressively slowing down the roller speed from 11 to 5 rpm without changing the steam pressure. The protein content of these whole-milk powders (N × 6.38) varied between 25 to 27%.

Lysine deterioration and hence its availability were measured according to the *in vitro* digestion procedure described by

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[1] In percentage composition: corn starch, 45; glucose, 18; dehydrated liver, 2; skim milk powder, 35; and adequate amounts of salts and vitamins.

Mauron et al. ('55). Spray-dried milk was taken as a standard of reference since its lysine availability has been found to be the same as in fresh milk. Total lysine content in fresh milk has been found to be 8.3% (Mauron et al., '55). The lysine availability in the milk powders tested is given in table 1. For most powders lysine destruction and inactivation are listed separately, but it has been shown (Mauron and Mottu, '58) that all the deteriorated lysine is unavailable to the rat for growth. Other essential amino acids are not deteriorated in roller-powders, as shown by Mauron et al. ('55) and by Mauron and Mottu ('58), with the exception of methionine, the availability of which is slightly reduced (10 to 20%) in roller-powders.

The diet consisted of the following percentage composition: whole-milk powder, 40; sucrose, 55; salts, 3; (McCollum and Davis, '14); dehydrated liver, 2. The diet was supplemented with the following vitamins in milligrams per 100 gm of ration: thiamine, 0.2; riboflavin, 0.3; pyridoxine, 0.3; Ca pantothenate, 1.0; choline chloride, 200; inositol, 4; tocopheryl acetate, 3; 286 I.U. vitamin A and 43 I.U. vitamin D.

Rats of our caries-susceptible Wistar strain were used. The experiment was started immediately after weaning when the rats weighed about 26 gm and was continued 20 days. Six groups of 10 animals each were used. Groups 1 to 3 were fed ad libitum, whereas groups 4 to 6 were pair-fed. Water was always provided ad libitum. The animals were housed in tinned wire cages in air-conditioned rooms. Food was administered in fine powder from a jar designed to prevent spilling.

Growth was recorded twice a week. Caries diagnosis was made according to the method of Mühlemann et al. ('56), which allows caries detection at a very early stage. Three degrees of carious lesions are distinguished, according to severity.

RESULTS

The results are shown in table 2. The growth of the animals declined in proportion to lysine deterioration. Growth of the groups fed ad libitum agrees well with that of the corresponding pair-fed groups; in both cases growth decreased with increasing lysine deterioration.

The caries incidence of the ad libitum (groups 1 to 3) as well as of the pair-fed groups (4 to 6) shows no correlation with growth or lysine deterioration.

DISCUSSION

In our experiment with caries susceptible rats, it has been found that lysine deterioration, i.e., lysine deficiency, did not cause or increase caries although growth and, therefore, the formation of the skeleton were seriously impaired. Bujard ('59), using the same milk powders, showed that lysine deterioration seriously disturbs endochondral ossification, delaying the appearance and growth of the nuclei of epiphysial

TABLE 1

In vitro lysine availability in milk powders tested

	Destruction[1]	Inactivation[2]	Deterioration[3]	Availability[4]
	%	%	%	%
Standard spray-dried milk powder 1 and 2	—	—	0	100.0
Roller powder A	14.6	20.0	34.6	65.4
Roller powder B	18.0	28.0	46.0	54.0
Roller powder C	17.0	29.3	46.3	53.7
Roller powder D	—	—	63.0	37.0
Roller powder E	27.0	42.0	69.0	31.0
Roller powder F	26.6	45.8	72.4	27.6

[1] Difference between lysine content of fresh milk and that of the sample, determined after acid hydrolysis expressed in per cent of lysine content of fresh milk.

[2] Difference between lysine deterioration and destruction.

[3] Difference between amount of lysine liberated enzymatically from fresh milk and that freed from the dried milk, expressed in per cent of lysine liberated from fresh milk.

[4] Lysine freed enzymatically from the sample expressed in per cent of lysine liberated from fresh milk.

TABLE 2
Growth and carious lesions

Group	Milk	Lysine deterioration	Weight gain	Degrees of carious lesions, average values[1]		
				A[2]	B[3]	C[4]
		%				
1	Spray powder 2	0	1.45 gm/day	18.0 ± 0.55[5]	8.1 ± 0.59	4.1 ± 0.52
2	Roller powder B	46	0.78 gm/day	15.5 ± 0.67	7.2 ± 0.58	3.0 ± 0.65
3	Roller powder E	69	0.29 gm/day	15.7 ± 0.40	7.7 ± 0.80	2.8 ± 0.84
4	Spray powder 2	0	1.03 gm/day/ 5 gm feed	16.5 ± 1.23	5.5 ± 0.75	0.9 ± 0.28
5	Roller powder B	46	0.63 gm/day/ 5 gm feed	16.0 ± 0.84	5.7 ± 0.37	1.4 ± 0.48
6	Roller powder E	69	0.36 gm/day/ 5 gm feed	15.5 ± 0.67	4.8 ± 0.40	1.2 ± 0.50

[1] According to Marthaler, Muehelmann, and Koenig ('56).
[2] Initial enamel caries.
[3] Enamel caries, reaching the dentino-enamel junction, initial dentin caries.
[4] Progressive dentin caries, cavitation.
[5] Standard deviation.

osteogenesis in the apophysis of the femur. Likins, Bavetta and Posner ('57) also presented data which indicate that a dietary lysine deficiency was associated with a decrease in the skeletal deposition of radiocalcium due to a failure in bone growth. This discrepancy between the lysine effect on caries and on skeletal growth is worth being mentioned.

Our results differ from those of McClure obtained with heated skim-milk powder, where caries frequency increased with the heat treatment. This may be due to the different strain of rats used or to the fact that our diet contained about 11% of butter fat, whereas McClure's diet was practically devoid of fat.[2] The complete absence of any correlation between lysine deterioration and caries in our experiments favor the hypothesis that in McClure's experiment with heated skim milk powder, it was not lysine, but another factor connected with the heat treatment which was involved. This would agree with another report by McClure ('52), where an increased caries incidence was found in rats fed a diet containing processed cereal foods. Here again, the processing of the cereal foods may have destroyed some caries-preventing substance or caused the formation of cariogenic compounds. It should also be noted that even in the experiments where McClure observed a certain caries-preventing effect of lysine, no proportionality could be found between the growth-promoting and the caries-preventing effect of lysine (McClure and Folk, '55b). If there should have been a difference in caries frequency between the different groups we are sure to have found it even using the short-term technique. The experiments published by Mühlemann and co-workers ('56) and our experience have proved the technique reliable.

It is concluded that at the present time our knowledge is not sufficient to attribute the caries-enhancing effect of heat treatments to a specific factor. Our results with diets of known available lysine content are in favor of the view that lysine deficiency *per se* is not an essential factor. This does not exclude the possibility that this deficiency may be an accessory factor under given circumstances.

SUMMARY

A series of whole-milk powders with increasing lysine deterioration was prepared by intensifying the heat treatment during roller-drying. Lysine availability in the powders was measured by *in vitro* diges-

[2] It may be mentioned here that McClure was studying smooth surface caries whereas we were concerned with fissure caries. It is possible that the mechanism may be different in the development of smooth surface caries and fissure caries.

tion. Diets containing 40% of these milk powders were fed to young rats, and caries incidence and growth determined. No relationship whatsoever could be found between caries incidence and lysine deterioration, whereas growth was progressively reduced as lysine deterioration increased.

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Thiamine Deficiency and the in vivo Oxidation of Lactate and Pyruvate Labeled with Carbon[14] [1]

JAMES H. JONES AND ELIZABETH DE ANGELI
Laboratory of Biochemistry, School of Veterinary Medicine and Department of Biochemistry, School of Medicine, University of Pennsylvania, Philadelphia

The work of Peters and associates, of Lohman and Schuster and of others[2] has shown that thiamine, as the pyrophosphate, is an essential part of the enzyme complex which decarboxylates pyruvic acid in biological systems. It is generally considered that this same cofactor is necessary for the enzymatic oxidation of α-ketoglutaric acid to succinic acid (Barron et al., '41; Stumpf et al., '47; Sanadi et al., '52; Reed and DeBusk, '52). Wright and Scott ('54), however, have found that a thiamine deficiency which decreases the *in vitro* rate of oxidation of pyruvate has little effect on the *in vitro* rate of oxidation of α-ketoglutarate. It has also been shown that during thiamine deficiency there is an increase in the amount of lactic acid and/or pyruvic acid in the blood (Thompson and Johnson, '35) and other tissues (Fisher, '31; Kinnersley and Peters, '30) of animals. Likewise there is an increased urinary excretion of pyruvate under the same conditions (Banerji and Harris, '39; Shils et al., '41). These observations have been interpreted as an indication of decreased pyruvate decarboxylation in thiamine deficiency, presumably as a result of a lower concentration of thiamine pyrophosphate in the cells. However, it should be noted that the quantity of pyruvate which is excreted is small when compared with the total amount which must be produced. For example, Shils, Day and McCollum ('41) found that the maximum excretion by the rat of bisulfite-binding compounds (expressed as pyruvic acid) was not over 10 mg per day, whereas food consumption at the same time was from 8 to 10 gm. Even if the food intake is reduced to one gram per day, 10 mg would account for only a small part of the pyruvate formed on a high carbohydrate diet. Most of the work on the relation of thiamine to the oxidation of alpha ketoacids has been done with micro-organisms, isolated tissues or purified enzyme systems.

In many of these studies, the experimental conditions were such that the cocarboxylase was the limiting factor, and any change from the normal level would be detected. A dietary deficiency of thiamine causes a decrease in the amount of thiamine pyrophosphate in the tissues which results in a reduction in the rate of decarboxylation of pyruvate when measured *in vitro* under the above conditions; but this diminution in the rate of pyruvate oxidation is not necessarily the cause of the deficiency signs and subsequent death of animals deprived of thiamine, since decarboxylation was not generally measured, and the conclusions were based solely on oxygen uptake.

At the time this work was started, no direct quantitative studies had been made on the effect of a thiamine deficiency on the rate of pyruvate oxidation by the intact animal. Shortly after our first preliminary communication,[1] Guggenheim and Olson ('53) reported on the effect of a dietary deficiency of thiamine on the *in vivo* oxidation of 2-C^{14} pyruvate. Their re-

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[1] Preliminary reports of this work have been presented: Jones, J. H. 1958 Thiamine deficiency and oxidation of 2-^{14}C pyruvate. IV Int. Cong., Biochem., p. 93 (abstract); Jones, J. H., and E. de Angeli 1952 Oxidation of lactate and pyruvate by thiamine deficient mice. Federation Proc., *11*: 237 (abstract).

[2] For a review of the early work on the relation of thiamine to the decarboxylation of pyruvic and other α-ketoacids see Jones, J. H. in Wohl's Dietotherapy, W. B. Saunders Company, Philadelphia, 1945.

sults were in general agreement with those reported here; however they did not report the specific activity of the carbon dioxide. It is possible that the deficient animal can still metabolize rather large amounts of pyruvate although the state of the deficiency is well advanced. In the following experiments this possibility has been tested by studying the *in vivo* oxidation of carbon[14]-labeled lactate and pyruvate by thiamine-deficient mice and comparing the results with those obtained from pair-fed controls. The deficiency was produced by feeding a diet lacking in thiamine and also by thiamine inhibitors.

EXPERIMENTAL

Dietary deficiency experiments. White mice of both sexes (predominantly male) obtained commercially, served as experimental animals. They were from one to two months old and most of them weighed 20 gm plus or minus 5 gm at the start of an experiment. The diet of the control animals had the following composition expressed in grams per kilogram: vitamin-free casein, 180; sucrose, 738; cottonseed oil, 20; salt mixture no. 12 (Jones and Foster, '42), 40; cellulose, 20; and choline chloride, 2.0. A few drops of oleum percomorphum were given to each animal once a week. The diet contained the following additional vitamins in milligrams per kilogram of ration: thiamine·HCl, 5.0; riboflavin, 5.0; pyridoxine·HCl, 5.0; Ca pantothenate, 30; nicotinic acid, 30; inositol, 30; pteroylglutamic acid, 1.0; biotin, 0.2; and 2-methyl-1,4-naphthoquinone, 2.5. The deficient diet was the same as above except that it contained no added thiamine. The animals not receiving thiamine showed the following deficiency signs: they lost appetite, lost weight, became weak, developed characteristic curvature of the spine, some dragged their hind quarters and some became moribund and an occasional one died. In agreement with earlier reports (Jones et al., '45; Morris, '47), no specific polyneutric symptoms were observed in the mice on the diet containing no added thiamine.

The animals were divided into pairs with both members of each pair of the same sex and litter. The pair-feeding technique was used throughout.

When an animal fed the incomplete diet became severely deficient, the food cup was taken out of the cage at 5 P.M. The next morning 37 mg (1.0 mmole of carbon) of the radioactive compound was given by stomach tube in 0.15 ml of aqueous solution. Respiratory carbon dioxide was collected continuously for three hours. At hourly intervals, the collecting tube of sodium hydroxide solution was changed. The carbonate was precipitated as the barium salt, washed, dried and weighed. The specific activity was subsequently determined and all counts calculated to infinite thickness. The next day the corresponding control animal was treated in a similar manner.

Oxidation of lactate-1-C[14]. In the first experiment the sodium salt of carboxyl-labeled lactic acid[4] (2,900 counts per minute per mg of carbon or 34,800 total counts) was administered, and the results are summarized in table 1. There were 8 mice in each group originally, but one of the deficient animals was accidentally killed during the administration of the lactate, one died during the second hour of the collection and another at the end of the second hour. The latter two died apparently from lack of thiamine. Neither in counts per minute per milligram nor in total counts expired during each collection period, was there a significant difference[5] ($P < 0.05$) between the deficient and control animals.

[4] The authors are grateful to Dr. Adelaide M. Delluva and Dr. Frank Eisenberg, Jr., for the synthesis of the lactates used in these experiments.

[5] The data from these experiments were subjected to two forms of statistical analyses. In the first the deficient animals of any one experiment were considered as comprising one population and the control animals of the same experiment as another population. The standard error of the mean was determined from the data obtained from each population and from these the standard error of the difference between the two means was calculated. All available data were included in these analyses.

In the second form of analysis the animals were considered as a series of pairs, and for any set of data the standard error of the mean of the differences between the values obtained for the two members of each pair was calculated. In the latter analyses, if an animal died before complete data were obtained, the results from the paired control were omitted from this point.

TABLE 1

The oxidation of C^{14}-labeled lactate and pyruvate by thiamine-deficient mice

Time period	Deficient animals			Control animals		
	No. of animals	Counts/ min./mg C	Total counts/min.	No. of animals	Counts/ min./mg C	Total counts/min.
Hour						
			Oxidation of sodium lactate-1-C^{14}			
1st	7	363 ± 44[1]	5135 ± 583	8	335 ± 47	5555 ± 979
2nd	6	378 ± 57	4185 ± 351	8	285 ± 45	4267 ± 549
3rd	5	195 ± 75	2299 ± 864	8	168 ± 35	2325 ± 544
			Oxidation of sodium lactate-2,3-C^{14}			
1st	6	236 ± 54	2845 ± 749	8	276 ± 35	4261 ± 542
2nd	6	377 ± 35	5087 ± 825	8	404 ± 14	6886 ± 799
3rd	6	383 ± 67	5053 ± 628	8	328 ± 40	5689 ± 574
			Oxidation of sodium pyruvate-2-C^{14}			
1st	10	720 ± 144	7264 ± 1278	9	689 ± 153	11,129 ± 2261
2nd	9	505 ± 103	4382 ± 474	9	434 ± 124	8025 ± 1421
3rd	9	343 ± 58	3112 ± 575	9	135 ± 30	2750 ± 652

[1] Standard error of the mean.

Oxidation of lactate-2,3-C^{14}. It is known that the carbon dioxide of the carboxyl group of lactic acid can be replaced by the carbon dioxide of the tissue cells. There was the possibility that in the above experiment this exchange (via pyruvic, malic and oxalacetic acids) took place to a significant degree, and that the evaluation of the radioactivity in the expired carbon dioxide was not a measure of the oxidation of the administered lactate. It was with this in mind that the next experiment was conducted in which the oxidation of sodium lactate-2-3-C^{14} by thiamine-deficient mice was studied. The proper functioning of the tricarboxylic acid cycle presumably is necessary for the biological oxidation of the radioactive carbons of this compound; consequently the use of lactate labeled in positions 2 and 3 might give some information concerning the acivity of pyruvic acid decarboxylase and of α-ketoglutaric acid oxidase in these animals. The lactate had an activity of 2400 counts per minute per mg carbon, and a total of 28,800 counts was administered. The results of this experiment are also presented in table 1.

Originally there were 8 animals in each of the two groups, but two of the animals not receiving thiamine died from the deficiency during the night previous to the day on which the collection of carbon dioxide had been planned. None died during the collection period. In this experiment, as in the first, there was no significant difference in either counts per milligram or in total counts between the two groups of animals (with and without thiamine). However, there appears to have been a difference between these two experiments in respect to the rate at which the labeled carbon was released. In the first experiment a tendency was noted toward a decreasing radioactivity from period to period in both deficient and control animals. In the second experiment the opposite tendency was evident. This difference is to be expected if the normal pathway of metabolism is being followed, for the carbon dioxide of the carboxyl group would be released by decarboxylation before the second and third carbons. From the standpoint of the relative rates at which labeled carbon dioxide was liberated by the deficient and control animals from lactate 1-C^{14} and lactate 2-3-C^{14}, the data from this experiment not only confirm the findings in the first experiment, but also indicate that the tricarboxylic acid cycle was functioning in a near normal manner in the thiamine-deficient mice.

Oxidation of pyruvate -2-C^{14}. To test the possibility that the above results were due to the oxidation of lactate by some pathway other than through pyruvic acid, the oxidation of pyruvate-2-C^{14} was investigated in the next experiment. This compound had an activity of 3150 counts per

minute per mg of carbon or a total count of 37,800. The results of this experiment are summarized in table 1. At the start there were 10 pairs of animals, but one of the controls did poorly and was destroyed, and one of the deficient animals died during the second hour of collection.

Although the activities of the carbon dioxide were higher than in either of the experiments with lactate, the results in general were about the same. The specific activity during the third hour for the deficient animals was significantly[6] higher than for the controls when calculated by the non-paired technique. This difference was probably due to the large amount of pyruvate metabolized by the controls during the first two hours which left less to be oxidized in the third hour. The controls showed significantly higher total counts than the deficient animals for the second period when calculated by either method.

The data as a whole from the three experiments described above show that there was practically no difference in *specific activities* (counts per minute per milligram of C) of the expired carbon dioxide between the deficient and control groups. In absolute amounts, as shown by total counts, the controls were consistently higher than the deficient animals. These results will be discussed further in connection with subsequent experiments. In the case of the two animals of the first experiment which died during the collection period, the counts per milligram for the last full hour that carbon dioxide was obtained were 404 and 497 as against 434 and 373 for the respective controls. The corresponding total counts were 4,572 and 3,965 as compared with 4,804 and 6,424. The second of these two deficient animals died at the end of the second hour, so the values given here are for the last 60 minutes that this animal was alive. Even though these animals were dying because of the deficiency of thiamine, no change in the relative rates at which lactate and pyruvate were oxidized occurred as shown by the counts per minute per milligram. It is highly improbable that the death of these animals was caused by the inability of the body cells to decarboxylate pyruvic acid.

Short-time collections of carbon dioxide. A further attempt was made to test the possibility that the liberation of the radioactive carbon dioxide was due to virtually complete randomization coupled with the exchange reaction between pyruvate and oxalacetate or malate, and not the result of decarboxylation and subsequent oxidation of the acetate by the tricarboxylic acid cycle. If this were true, it is possible that the carbon dioxide collected for short periods of time, insufficient to allow for randomization, might show a greater difference in activity between deficient and control animals. Experiments to test this hypothesis were conducted, as described earlier, on mice. The diet was similar to that previously used except that the amounts of all the 13 vitamins were doubled, and glycerol was added at a level of one per cent to reduce the probability of oxidative destruction of dietary essentials (Kandutsch and Baumann, '53). No thiamine was added to the deficient diet. Eighteen milligrams of sodium pyruvate-2-C^{14} containing a total of 6,000 counts per minute were injected intraperitoneally. Collection of carbon dioxide was started immediately and continued for 40 minutes in periods of 5, 5, 10 and 20 minutes.

The average activities in counts per minute per milligram of carbon dioxide carbon and the total counts for each time period are given in table 2. One of the control animals did poorly from the start of the experiment and was finally sacrificed. As can be seen, the specific activities as well as the total counts were very low for the first period; consequently, the error is undoubtedly large. Although the controls consistently gave slightly higher counts in respect to specific activity and to total count, none of the differences was significant when calculated as nonpaired data. When considered as pairs, however, the difference between averages was highly significant for the first period, just significant for the second period, nonsignificant for the third period and almost identical values for the two groups were obtained for the fourth period with a "t" value approaching zero.

[6] See footnote 5, p. 538.

TABLE 2

Short-time collections
Oxidation of sodium pyruvate-2-C¹⁴

Time period	Deficient animals			Control animals		
	No. of animals	Counts/ min./mg C	Total counts/min.	No. of animals	Counts/ min./mg C	Total counts/min.
minutes						
0 to 5	8	3.12 ± 0.89[1]	4.25 ± 1.91	7	4.84 ± 0.88	6.22 ± 0.94
5 to 10	8	7.28 ± 1.53	10.93 ± 4.40	7	9.48 ± 1.52	13.30 ± 3.13
10 to 20	8	18.52 ± 2.62	43.39 ± 10.5	7	22.62 ± 4.26	65.06 ± 12.0
20 to 40	8	27.40 ± 4.48	110.06 ± 29.8	7	28.88 ± 6.67	122.33 ± 40.9

[1] Standard error of the mean.

These results would seem to indicate that some randomization was taking place which was followed by liberation of carbon dioxide through the exchange reaction. It should be pointed out, however, in order to randomize pyruvate-2-C¹⁴ so as to bring the labeled carbon into the carboxyl group, it is necessary to convert this compound into oxalacetate or malate followed by either direct equilibration with fumarate and then once around the tricarboxylic acid cycle. Or, if it does not equilibrate directly with fumarate, it must go around the tricarboxylic acid cycle twice. As essentially no difference was found in the activity of the carbon dioxide produced by deficient and control animals at the end of 40 minutes, it must be assumed that randomization was complete in this length of time if the results are to be explained by this means. However, at the end of this time, namely 40 minutes, only 3% of the radioactive carbon had appeared as carbon dioxide. On the basis of the known pathways for pyruvate metabolism it is impossible that the pyruvate could be completely randomized in this way, since only 3% of the radioactive carbon had been released as carbon dioxide. Therefore, these results must have been the result of some cause other than randomization.

Inhibitor experiment.[2] Various investigators (Woolley, '51; Naber et al., '54; Eich and Cerecedo, '54) have reported that the deficiency signs produced in experimental animals by oxythiamine and pyrithiamine, two antithiamine compounds, are quite different. Oxythiamine, given to mice, causes a marked anorexia, with rapid loss of weight terminating in death without the animals showing signs of polyneuritis. This is much the same as the symptoms produced in many animals by withholding thiamine from the diet. Pyrithiamine, on the other hand, frequently produces typical polyneuritic signs. This being the case, it was of interest to compare the effects of these two inhibitors on the oxidation of pyruvate with each other and with the above data obtained with the dietary deficiency. The details of these experiments were very much the same as those described in connection with the "short-time" collection except that the collection of carbon dioxide was continued for two hours and divided into 5 periods of zero to 5, 5 to 15, 15 to 30, 30 to 60 and 60 to 120 minutes. The diet of the "deficient" mice contained the same amount of thiamine as that of the controls. To save on the amount of inhibitors necessary, the thiamine was given at a level of one milligram per kilogram of diet. This quantity allows mice to grow at an approximately normal rate. The oxythiamine was given at a ratio of 50 to one of thiamine and the pyrithiamine at a 25 to one ratio. The animals receiving oxythiamine were continued until they were critically deficient, whereas the mice receiving pyrithiamine were taken for experiment as soon as they showed definite signs of polyneuritis.

The results of these two studies are presented in table 3. The amount of carbon dioxide collected during the 5-minute period from two of the mice receiving oxythiamine and from one mouse receiving pyrithiamine was too small to give even approximately reliable results. In all periods with oxythiamine, the average count per

[2] See footnote 5, p. 538.

TABLE 3

Oxidation of sodium pyruvate-2-C^{14} by mice made deficient by thiamine inhibitors

Time period	Deficient animals			Control animals		
	No. of animals	Counts/min./mg C	Total counts/min.	No. of animals	Counts/min./mg C	Total counts/min.
minutes						
		Deficiency produced by oxythiamine				
0 to 5	6[2]	1.84 ± 0.34	1.60 ± 0.92	7[2]	2.26 ± 0.24	4.05 ± 0.64
5 to 15	7	9.24 ± 0.97	23.9 ± 5.51	8	10.4 ± 1.03	44.3 ± 6.62
15 to 30	7	15.6 ± 1.91	53.6 ± 11.1	8	15.79 ± 1.24	93.2 ± 11.2
30 to 60	7	16.4 ± 2.90	122.7 ± 12.9	8	12.03 ± 1.28	146.7 ± 18.1
60 to 120	7	16.3 ± 3.58	206.0 ± 23.1	8	11.02 ± 1.21	269.8 ± 41.0
		Deficiency produced by Pyrithiamine				
0 to 5	6[1]	2.01 ± 0.40[3]	3.46 ± 0.38	6	1.69 ± 0.27	2.77 ± 0.46
5 to 15	8	8.71 ± 1.01	29.9 ± 4.37	6	9.13 ± 1.31	32.7 ± 6.23
15 to 30	8	13.16 ± 1.16	70.5 ± 7.25	6	18.1 ± 3.52	94.8 ± 17.1
30 to 60	7	11.19 ± 1.11	111.1 ± 16.1	6	20.8 ± 4.22	192.8 ± 36.2
60 to 120	6	7.78 ± 1.15	140.6 ± 38.5	6	20.6 ± 4.42	409.8 ± 101.7

[1] With two animals in the first period there was insufficient carbonate for plating.
[2] Standard error of the mean.
[3] With one animal in the first period there was insufficient carbonate for plating.

minute per milligram was higher for the controls than for the deficient animals, but in no period was the difference statistically significant. In respect to total counts, there was a still greater difference between control and deficient animals. When these data were treated as pairs, the difference was significant in the second and 5th periods and close to being significant in the 4th period. The difference in the 5th period was also significant by the non-paired calculation. Since the specific activities in this experiment were higher for the control than deficient animals in all periods, and since the differences in total counts were larger than in the other experiments, it is possible that oxythiamine has specificially inhibited the oxidation of pyruvate to a limited extent.

When the deficiency was produced by pyrithiamine, none of the differences in specific activity (counts per milligram) even approached values which indicated significance. It should be noted that for each of the first two periods the average for the deficient animals was higher than for that of the controls. In regard to total counts, again the control animals showed consistently higher values than the deficient animals, but the difference was significant for the third period only and only when considered as nonpaired.

In figure 1, the "t" values for the two inhibitor experiments, and calculated for the counts per milligram by the paired technique, are plotted against time in minutes. It can be observed that in the oxythiamine experiment the "t" value increased from 0.312 in the first period to 1.570 in the second and to 2.061 in the third. This is opposite to that obtained in short-period collections on the dietary deficiency of thiamine and opposite to that expected if randomization is taking place.

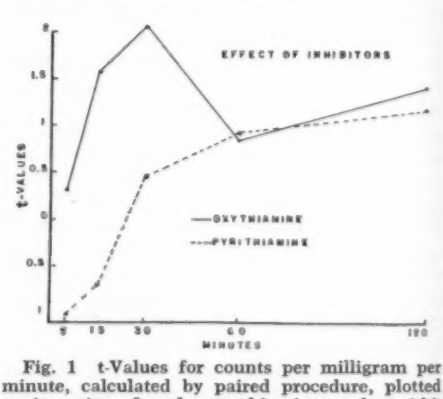

Fig. 1 t-Values for counts per milligram per minute, calculated by paired procedure, plotted against time for the oxythiamine and pyrithiamine experiments. (Values below zero indicate that the average for the deficient animals was higher than for the controls.)

There was also no significant difference at any period when the deficiency was produced with pyrithiamine. Here again the general trend of the change is opposite to

that expected if randomization were taking place; that is, randomization necessary to allow radioactive carbon from pyruvate-2-C^{14} to be freed by the decarboxylation of oxalacetate or malate. In neither of the two experiments with the inhibitors was there any evidence that this type of randomization was occurring.

These data taken as a whole throw considerable doubt on the possibility that the radioactivity of the respiratory carbon dioxide can be accounted for by randomization and the exchange reaction.

DISCUSSION

In the interpretation of the above results two concepts must be considered; one based on counts per milligram per minute or specific activity and the other based on the total counts. The specific activity is a measure of the ratio of the carbon dioxide arising from the labeled lactate or pyruvate to the total carbon dioxide produced. If the oxidation of pyruvate is significantly and specifically inhibited through failure of decarboxylation, the specific activity of the carbon dioxide from the animals in which inhibition is taking place (thiamine-deficient) would be less than that from animals in which there is no inhibition.

In all, 23 comparisons were made in this respect and in 9 cases the average for the deficient animals was higher than for the corresponding controls, and in 14 cases the opposite was observed. In 7 of these comparisons there was a difference of only 1.2 to 8.5%. Five of the 14 comparisons in which the controls had the higher average were in the oxythiamine experiment. As it is possible that this antivitamin exerted a slight but specific inhibition on the oxidation of pyruvate, it would seem proper to omit this experiment when the others are being considered. There are then 9 comparisons in which the deficient animals had a higher specific activity than the corresponding controls and 9 cases in which it was the opposite. These results show clearly that the fraction of total carbon dioxide coming from the labeled compounds is essentially the same in deficient and control animals of the dietary-deficient and pyrithiamine experiments. This can only mean that in these experiments the oxidation of lactate and pyruvate was not being *specifically* inhibited by these deficiencies.

In contrast to specific activity, the total counts were consistently higher, 17 of 18 comparisons, in the controls than in the deficient animals (omitting again the oxythiamine animals). This was as expected. At the time the carbon dioxide was collected the mice not receiving thiamine were in the terminal stages of the deficient state. As such they were extremely ill, in some cases moribund, and it is not surprising to find that such animals had a lower total metabolism than the pair-fed, but otherwise healthy, active and hungry animals. In addition there was a difference in the voluntary movements of the animals. In general the deficient animals were definitely less active during the collections than were the controls. This would also increase the total metabolism of the controls in comparison with the deficient animals. Various modifications of the collecting procedure were tried, but none was successful in reducing the voluntary movements of the controls to that of the deficient animals. But in spite of the increased output of carbon dioxide the fractions coming from the labeled compounds were nearly identical in both groups of animals. This emphasizes the fact that a difference in total counts does not necessarily indicate a difference in the type of metabolism taking place, and by using labeled substrates it is possible to obtain comparative information on the pathways of metabolism even though total oxidation is not equal in the two groups. It appears that the lack of thiamine, produced by dietary means or by the use of oxythiamine or pyrithiamine, is effective at locales other than those involving the oxidation of pyruvate. Possibly some organs or tissues may become deficient in thiamine before the body as a whole, and the failure on the part of the animal is due to this more-or-less local deficiency. Wright and Scott ('54) have found that the decrease in oxygen uptake by tissues from thiamine-deficient rats, as compared with the same tissues from normal controls and with pyruvate as the substrate, was more pronounced with liver than with brain, whereas kidney and heart were intermediate.

It is also possible that in both the pair-fed control animals and in the deficient animals an unusually large part of the pyruvate enters the tricarboxylic acid cycle via the carboxylation of pyruvic acid to form a 4-carbon dicarboxylic acid. Evidence in favor of this view has been presented by Freedman and Graff ('58), who have shown that starvation increases the proportion of pyruvic acid which enters the tricarboxylic acid cycle through the formation of the dicarboxylic acids and conversely decreases that part entering the cycle as acetyl-CoA. This could result in equal rates of oxidation by the two groups of animals (controls and thiamine-deficient) if it is assumed that the thiamine deficiency does not impede the decarboxylation of α-ketoglutaric acid. If this explanation of the results presented here is correct, it must be presumed that starvation has the same effect on pyruvate oxidation as does a deficiency of thiamine, or that the effect of the vitamin deficiency on pyruvate metabolism is the result of inanition. Guggenheim and Olson ('53) have mentioned the latter as a possibility.

It is also true that during the collection of carbon dioxide these animals were confined to a small flask, and the conditions were approaching those of the basal state. Possibly under greater physical stress a larger difference between deficient and control animals, in their ability to oxidize these compounds, might have become evident. It may be that the maximum rate at which a thiamine-deficient animal can decarboxylate pyruvate can be reduced to a basal level below which it will not fall even in the animal dying from the deficiency. However, it is known that thiamine, in addition to its role in the decarboxylation of pyruvate and α-ketoglutarate, is a necessary coenzyme (as the pyrophosphate) for the metabolism of pentose phosphate (Horecker and Smyrniotis, '53; Racker et al., '53). Several groups of workers have also demonstrated in various ways that the derangements in metabolism produced by oxythiamine and pyrithiamine are not identical. Eich and Cerecedo ('54) have also shown with a partially purified enzyme system that neither oxythiamine nor pyrithiamine inhibited the decarboxylation of pyruvate. Woolley and Merrifield[7] believe that thiamine serves some other biological functions "not mediated through cocarboxylase." These authors have suggested that the signs of a thiamine deficiency may be due to the inhibition of reactions other than the decarboxylation of pyruvate and α-ketoglutarate. The present data provide strong additional evidence for the correctness of this concept. With the possible exception of the oxythiamine animals, no differential impairment on the part of thiamine-deficient mice in their ability to oxidize both lactate and pyruvate was observed under the conditions of these experiments.

The aforementioned data also discount the possibility that these results are due to randomization followed by the release of carbon dioxide through the exchange reaction between pyruvate on the one hand and malate and/or oxalacetate on the other.

SUMMARY

Each of three groups of thiamine-deficient mice was given one of the following: lactate-1-C^{14}, lactate-2, 3-C^{14} or pyruvate-2-C^{14}, and the activity of the respiratory carbon dioxide was determined. Each deficient animal had a pair-fed control which received the same labeled compound. Respiratory carbon dioxide was collected for three hours at hourly intervals. The average specific activity of the expired carbon dioxide was essentially the same in deficient and control animals for each hour and for all three experiments. The total carbon dioxide and hence the total count was consistently higher for the controls than for the deficient animals but in no case was the difference statistically significant.

In another experiment, in which pyruvate-2-C^{14} was used, the carbon dioxide was collected in 5, 5, 10 and 20-minute periods. The amounts and activities of the carbon dioxide for the first two periods were very low, but the specific activity for these two periods was significantly greater for the controls when the data were treated as paired but not when treated as unpaired. The differences between activities for the

[7] Woolley, D. W., and R. B. Merrifield 1952 Evidence for a metabolic function of thiamine not mediated through cocarboxylate. Federation Proc., *11*: 458 (abstract).

other two periods were not significant. Oxidation of the pyruvate by the deficient and control groups in this experiment was slight (about 3%).

No significant differences were noted in the specific activities when the deficiency was produced with either oxythiamine or pyrithiamine. However, the averages in the oxythiamine experiment were higher for the controls in all periods. Oxythiamine produced a significant difference in total counts in the second and fifth periods. Possibly oxythiamine specifically inhibited the oxidation of pyruvate to a significant but limited extent. In the pyrithiamine experiment the controls were significantly higher in total counts in the third period when the data were considered as nonpaired.

By considering the specific activity and total count it is possible to compare the deficient and control groups in respect to the percentage of total carbon dioxide which originates in the labeled compound, regardless of the total amount of carbon dioxide expired. The significance of these results is discussed from this point of view. These data negate the possibility that the production of carbon dioxide from pyruvate by thiamine-deficient mice is due to randomization, and strongly support the thesis that the symptoms and signs of thiamine deficiency as seen in laboratory mammals are not the result of a specific failure on the part of the intact animal to decarboxylate pyruvate.

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ADDENDUM

Since this paper was presented for publication, Koeppe et al. (R. E. Koeppe, G. A. Mourkides and R. J. Hill 1959 J. Biol. Chem., *234:* 2219) have reported, in agreement with Freedman and Graff ('58) that fasting of rats decreased profoundly the conversion of pyruvate to acetyl-CoA, and in addition they found that thiamine-deficient animals "decarboxylated at least as great a percentage of pyruvate to acetyl-CoA as did the well fed normal animal."

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Unidentified Nutrients Required by the Hyperthyroid Rat

LESLIE P. DRYDEN, GEORGE H. RIEDEL AND ARTHUR M. HARTMAN
Dairy Cattle Research Branch, Animal Husbandry Research Division, ARS, Agricultural Research Center, Beltsville, Maryland

When weanling rats are fed certain purified rations containing thyroactive substances, their growth rate is diminished and their survival impaired. Improvement in both growth and survival has been obtained in many instances (Drill, '43; Betheil and Lardy, '49; Register et al., '49) by increasing the amounts of known nutrients in the ration beyond those normally required by the rat. Still further improvement in both respects has been observed frequently when certain crude food materials were added to the ration (Ershoff and Hershberg, '45; Ershoff, '47, '50a, '50b; Betheil et al., '47; Betheil and Lardy, '49; Stevens and Henderson, '58; Overby et al., '59a). Such a result has at times been attributed (Ershoff, '49) to the presence in these foods of unidentified nutrients which are normally, that is, under nonstress situations, either not required by the rat or else required only in amounts that can be supplied by synthesis.

Dried whole liver or liver residue has been used frequently as a source of such unidentified nutrients. Some workers (Ershoff, '49; Graham et al., '52) have found that under their conditions, such a food material has brought about normal growth in the hyperthyroid rat. In other instances (Westerfeld and Richert, '52; Stevens and Henderson, '58; Overby et al., '59a, b), improved, but not fully normal, growth has occurred. Results obtained in this laboratory fall in the latter category.

In this paper, we wish to indicate the extent to which our data agree and the extent to which they disagree with those of other workers and to present evidence suggesting the probable multiple nature of the unidentified factors required by the hyperthyroid rat for growth.

EXPERIMENTAL PROCEDURE

The animals used in these experiments were male albino rats from a colony descended primarily from the Wistar strain but maintained as a separate unit at Beltsville for more than 20 years. They were reared by stock mothers which in most instances were transferred at parturition to a purified casein-sucrose ration similar to that used in the experimental work except that it did not contain a thyroactive substance. The young rats were weaned at 25 days of age and started on experiment at about 28 days of age. In a few instances, stock weanling young were taken when 21 days old and placed on the purified ration for one week prior to starting on experiment. During the experimental period, the rats were maintained in individual cages provided with raised screen floors. Rations and distilled water were supplied ad libitum.

The basal ration used in these experiments had the following composition (per cent): sucrose, 59.93; alcohol-extracted casein (Hartman et al., '51), 24.85; salt mixture (Hawk and Oser, '31), 4.50; DL-methionine, 0.20; cottonseed oil, 9.85; fish liver oil,[1] 0.15; added vitamins,[2] 0.37;

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[1] Navitol with Viosterol (concentrated oleovitamin A and D) U.S.P., E. R. Squibb and Sons, New York. According to manufacturer, contained per gm 65,000 I.U. of vitamin A and 13,000 I.U. of vitamin D.

[2] Mg/100 gm ration: thiamine·HCl, 1.6; riboflavin, 1.6; pyridoxine·HCl, 1.6; Ca pantothenate, 10.0; choline chloride, 240.0; nicotinic acid, 10.0; inositol, 10.0; *p*-aminobenzoic acid, 60.0; biotin, 0.02; pteroylglutamic acid, 0.20; ascorbic acid, 10.0; α-tocopheryl acetate, 20.0; 2-methyl-1, 4-naphthoquinone, 0.5; and vitamin B_{12}, 0.01.

[3] Protamone, kindly supplied by Cerophyl Laboratories, Inc., Kansas City, Mo.; stated to contain 1.07% of thyroxine and about 7% of total iodine.

and iodinated casein,[3] 0.15. This level of iodinated casein was decided upon after preliminary experimentation and was used in this work, except where otherwise indicated. This level represented a compromise designed to achieve the best balance between the increasingly larger depression in weight gain, on the one hand, and the greater mortality on the other, which were associated with increasingly higher levels of thyroprotein. At this level, an average of approximately 78% of the rats fed the basal ration survived the experimental period.

Test supplements, unless otherwise indicated, replaced equal amounts of sucrose in the ration. Although some of these supplements improved survival on the basal ration, the criterion used has been the total weight gain during the experiment. An experimental period of three weeks was used, unless otherwise indicated. Comparisons within groups or experiments were based on litter-mates, a member of each litter having been assigned at random to each diet. Where one member of a litter died, the results from the entire litter were eliminated from the comparisons.

RESULTS AND DISCUSSION

The growth obtained with weanling rats fed the basal ration without thyroprotein is shown in table 1. Alterations in the composition of this ration such as the inclusion of a steroid (cholesterol), increasing the level of fat to 23%, substituting dextrin for sucrose or inclusion of an antibiotic (Aureomycin)[4] were all without significant effect on the rate of growth (groups 1 and 2). Likewise, the feeding of sources of possible unidentified nutrients, such as dried whole liver or fishmeal, was without effect (groups 3 and 4). When thyroprotein was incorporated in the ration, however, the growth rate was considerably depressed (group 5). Although not shown here, seasonal variations in the growth of both normal and hyperthyroid rats were observed that were generally similar to those described by Overby et al. ('59a).

When thyroactive casein-sucrose diets deficient in vitamin B_{12} were fed to stock weanling rats, growth responses with vitamin B_{12} were secured by some workers (Betheil and Lardy, '49) but not by others (Ershoff, '50a). In our tests (table 2), rats weaned from a vitamin B_{12}-deficient ration showed a slightly greater response to the vitamin in the absence of thyroprotein than in its presence. With thyroprotein, dried liver promoted more rapid

[4] American Cyanamid Company.

TABLE 1

Effect of alterations in the basal ration without thyroprotein on the growth of the normal rat

Group	Test material	No. of litters	Average 3-week gain in weight		t[1]
			Basal ration without thyroprotein	Basal ration without thyroprotein + test material	
			gm	*gm*	
1	Cholesterol (0.50%)			146	1.4
	Sucrose replaced by dextrin	8	144	146	0.5
	Fat increased to 23%[2]			136	0.9
2	Aureomycin[3] *(100 mg/kg)*	8	144	137	1.3
3	Dried whole liver (10%)	8	132	132	0.1
4	Fish meal (10%)	10	166	162	0.5
5	Iodinated casein (0.15%)	22	143	78	19.0**

[1] The symbol ** adjacent to or in connection with a t or F value indicates statistical significance at or less than the 1% level; * indicates significance at the 5% level or between the 5% and 1% levels; no * indicates no statistically significant difference (Snedecor, '56).
[2] Substituted isodynamically for sucrose.
[3] American Cyanamid Company.

TABLE 2

TABLE 2

Effect of vitamin B_{12} deficiency on the growth of the hyperthyroid rat

Group	Mother's diet during lactation	Iodinated casein fed	No. of litters	Average 3-week gain in weight					
				Basal ration without vitamin B_{12}	Basal ration	Basal ration + 10% dried liver[1]	t[2]	F[2]	Duncan's multiple range test[3]
		%		gm	gm	gm			
				(a)	(b)	(c)			
1	No vitamin B_{12}	0.00	8	94	132	132	—	12.0**	a/bc
2	No vitamin B_{12}	0.30	5	60	80	104	—	13.1**	a/b/c
3	No vitamin B_{12}	0.15	77	—	101	116	7.2**	—	—
4	Vitamin B_{12}	0.15	427	—	85	112	25.8**	—	—

[1] Either whole liver or liver residue.

[2] See footnote 1, table 1.

[3] LeClerg ('57); a particular alphabetical symbol is used to represent the mean listed in the column under that symbol. A given mean is significantly different (at the 5% level) from another mean in the same group if separated from it by a diagonal line.

growth than vitamin B_{12}, a finding in accord with that of Betheil and Lardy ('49).

In tests for unidentified nutrients in liver, smaller differences were obtained with vitamin B_{12}-deficient young (group 3) than with vitamin B_{12}-sufficient young (group 4). Thus even the initial vitamin B_{12} status of the hyperthyroid rat appears important.

The depression in growth obtained with the basal thyroprotein ration containing all known nutrients, including vitamin B_{12}, could be prevented partially by feeding crude food materials. Dried whole liver as a standard consistently improved growth. Thus, in 89% of the litters, the rat receiving liver gained on an average 15 to 32% more weight than its control litter-mate (groups 3 and 4, table 2).

Tests of dried whole liver and liver fractions are shown in table 3. The water-soluble fraction (liver concentrate) was completely inactive, whereas three of 4 lots of liver residue were quite active. Defatted dried liver was as potent as dried whole liver. These results agree with those of other workers (Ershoff, '47; Graham et al., '52; Tappan et al., '53; Overby et al., '59b). With the lots tested, the various brands of dried whole liver did not stimulate growth equally. Whereas Stevens and Henderson ('58) reported beef-liver residue to be less active than pork-liver residue, such differences apparently cannot account for the results observed here, since the lots of brand 2 and brand 3 dried whole liver used, which promoted less growth than brand 1, were both pork liver (groups 4 and 5). Brand 1[5] of dried whole liver was used as the positive control throughout the remaining tests in this paper. It was fed at a level of 10%, which in groups 6 and 7 promoted the maximum growth that could be obtained from this supplement.

Tests of certain plant food materials are shown in table 4. Cottonseed meal was the most active of these substances at the 10% level, the growth beyond that obtained on the basal ration averaging

[5] Desiccated Liver, N.F.; Wilson Laboratories, Chicago. This product is made from pork liver, beef liver or a mixture of the two, depending upon availability.

TABLE 3

Effect of dried liver and liver fractions on the growth of the hyperthyroid rat

Group	Test material		No. of litters	Average 3-week gain in weight			
	Nature[1]	Amount fed		Basal ration	Basal ration + test material	F[2]	Duncan's multiple range test[3]
		%		*gm* (a)	*gm* (b)		
1[4]	Liver residue, brand 1, lot 1	10	10	78	108	68.7**	a/b
2	Liver residue, brand 1, lot 2[5]	10	5	111	99	15.8**	ab/b_1
	Dried whole liver, brand 1	10			133(b_1)		
3	Liver residue, brand 1, lot 3[5]	10	5	100	141	7.6**	a/bb_1b_2
	Liver residue, brand 1, lot 4[5]	10			144(b_1)		
	Dried whole liver, brand 1	10			134(b_2)		
4	Liver concentrate, brand 2[6]	4	8	80	79	16.2**	ab/b_1b_2/b_3
	Dried whole liver, brand 2[5]	10			99(b_1)		
	Defatted dried liver, brand 2[5]	10			101(b_2)		
	Dried whole liver, brand 1	10			122(b_3)		
5	Liver concentrate, brand 3[7]	10	5	100	78	26.6**	a/b/b_1b_2/b_3
	Dried whole liver, brand 3[5]	10			122(b_1)		
	Defatted dried liver, brand 3[7]	10			122(b_2)		
	Dried whole liver, brand 1	10			137(b_3)		
6	Dried whole liver, brand 1	1	4	71	76	6.3**	abb$_1$/bb_1b_2/$b_2b_3b_4$
	Dried whole liver, brand 1	2			79(b_1)		
	Dried whole liver, brand 1	5			86(b_2)		
	Dried whole liver, brand 1	10			94(b_3)		
	Dried whole liver, brand 1	15			96(b_4)		
7	Dried whole liver, brand 1	10	8	—	109	1.5	—
	Dried whole liver, brand 1	15			103(b_1)		

[1] Liver residue is the heat-coagulated water-insoluble residue which is obtained in the preparation of liver concentrate, N.F., dried in vacuum and powdered. Liver concentrate, dried whole liver and defatted dried liver are, respectively, Liver Concentrate, N.F., Desiccated Liver, N.F., and Desiccated Liver (defatted), N.F.

[2] See footnote 1, table 1.

[3] See footnote 3, table 2.

[4] 0.30% Iodinated casein fed.

[5] Pork.

[6] Mixture of beef and pork.

[7] Beef.

81% of that found with liver. Stevens and Henderson ('58) found cottonseed meal to be inactive at this level. Distillers' solubles and dried grass promoted 77 and 69%, respectively, of the growth increment with liver. Dried brewers' yeast gave about one-half the activity of liver. Yeast has been variously reported to have considerable or slight activity (Betheil et al., '47; Ershoff, '48, '50b; Tappan et al., '53) or no activity at all (O'Dell et al., '55; Stevens and Henderson, '58) for growth. Corn meal gave negative results at levels of 10 or 20% but showed a pronounced growth stimulation at 40%. This agrees in general with the results obtained by other workers (Lewis et al., '50; Tappan et al., '53; Stevens and Henderson, '58). Soybean oil meal, when fed at 10%, was inactive. Such a result was also obtained by Stevens and Henderson ('58) but not by O'Dell et al. ('55) who found it to be moderately active. One brand of soy protein was completely without effect. Another brand, however, increased growth about 8% when fed at 10% of the ration. This same brand, when fed at 31.5%, gave growth that was at least as good as that obtained with dried liver. Emerson et al.[6] and Stevens and Henderson ('58) likewise found activity in soy protein.

Tests were also made with certain animal products, some of which are shown in table 4. The dairy products tested (dried whole milk, dried skim milk, cheddar cheese, dried cheese whey, casein and butter) were without activity. Dietrich et al. ('52), however, found crude casein and dried whey to be somewhat active. In agreement with Ershoff ('48) and Dietrich et al. ('52), fish solubles were found inactive. Fishmeal, on the other hand, was the most active substance tested at the 10% level (group 6, table 2). Ershoff ('50b) and Emerson and Folkers ('51) but not Stevens and Henderson ('58) found some activity in fishmeal.

In view of the conflicting results that have been obtained with regard to the apparent activity of some of the crude food substances tested, the question arises as to what extent, under our conditions, possible deficiencies of the basal ration in known nutrients could explain the increased growth obtained with these food substances.

Tests of the basal ration for adequacy in known nutrients are shown in table 5. Increases in vitamin content gave no increased growth. Increasing the amount of the salt mixture, altering its composition or feeding the ash of dried whole liver was also without significant effect.

Alterations in the kind or level of protein did not improve growth. Other tests were based on Harper's ('59) findings in regard to the limiting amino acids in casein for growth of the normal rat. Increasing or decreasing the methionine content (groups 8, 9) was without effect on growth. Its inclusion at 0.2%, as in the basal ration, appeared to improve survival slightly, in accordance with the findings of Boldt et al. ('58). In other tests, not shown, amino acids were added to the ration in amounts equivalent to their content in 5% of casein. The following combinations were tested: (1) threonine; (2) threonine, tryptophan, isoleucine and leucine; (3) glycine, alanine, tyrosine, proline, serine, cystine, aspartic acid and glutamic acid; (4) glycine and arginine; and (5) lysine. In no instance was there any effect on growth.

The feeding of antibiotics has been found to stimulate growth of the hyperthyroid rat (Meites and Ogle, '51; Stevens and Henderson, '58; Vogel et al., '58; Overby et al., '59a). In our work (table 5), Aureomycin (group 11) was ineffective and penicillin (group 12) failed to give a statistically significant increase.

A number of substances reported to promote the growth of rats or mice under certain conditions were tested and found to be without effect under our conditions. Such substances include lyxoflavin (30 mg/kg ration), orotic acid (1 mg/100 gm diet), various purines (adenine, guanine, xanthine, 0.05%), *L. bifidus* factor (fed as gastric mucin, 1% of ration) and thioctic acid (1 mg/kg ration).

[6] Emerson, G. A., B. Esser and A. C. Page 1956 Nutritional studies with rats subjected to thyrotoxic stress. Federation Proc., *15:* 549 (abstract).

TABLE 4

Effect on growth of adding crude food materials to the basal thyroprotein ration

Group	Test material		No. of litters	Average 3-week gain in weight				
	Nature	Amount fed		Basal ration	Basal ration + test material	Basal ration + 10% dried whole liver	F[1]	Duncan's multiple range test[2]
		%		*gm*	*gm*	*gm*		
				(a)	(b)	(c)		
1	Corn meal, yellow	10	14	98	93	115	8.4**	ab/c
2	Corn meal, yellow	20			99			
	Corn meal, yellow	40	4	99	118(b_1)	111	4.3*	abc/b_1c
3	Cottonseed meal[3]	10	8	95	121	127	21.1**	a/bc
4	Cottonseed meal[3]	10	9	86	100	—	6.4*	a/b
5[4]	Distillers' solubles	10	7	95	112	117	5.8*	a/bc
6	Fish meal	10	10	83	117	110	13.0**	a/bc
7	Fish solubles	4	6	85	78	106	11.6**	ab/c
8	Grass, dried[4]	10	8	95	117	127	18.6**	a/bc
9	Soybean oil meal	10	9	86	78	—	2.5	—
10	Soy protein, brand 1	10	19	85	92	—	5.7*	a/b
11[5]	Soy protein, brand 1	31.5[6]	8	93	120	116	10.4**	a/bc
12	Soy protein, brand 2	10	6	85	80	117	10.2**	ab/c
13[7]	Yeast, dried brewers'[8]	10	12	72	88	106	12.9**	a/b/c

[1] See footnote 1, table 1.
[2] See footnote 3, table 2.
[3] Prepared by screw-press method.
[4] Cerophyll.
[5] 0.20% Iodinated casein fed.
[6] Replaced all of casein and part of sucrose. Protein level equal to that of basal ration.
[7] 0.25% Iodinated casein fed to 4 litters, 0.15% to others.
[8] Anheuser-Busch, strain G.

TABLE 5

Effect on growth of adding known nutrients to the basal thyroprotein ration

Group	Test material	No. of litters	Average 3-week gain in weight			F[1]	Duncan's multiple range test[2]
			Basal ration	Basal ration + test material	Basal ration + 10% dried whole liver		
			gm (a)	*gm* (b)	*gm* (c)		
	Vitamins:						
1	All in mixture doubled	11	67	70	104	12.8**	ab/c
2	All in mixture increased 5×[3]	13	92	92	110	5.7**	ab/c
3	Vitamins A and D increased 2×[4]	4	68	72	94	3.9	—
	Minerals:						
4	Increased from 4.5 to 6.75%	6	97	92	126	16.2**	ab/c
5	Salt mixture no. 12[5] substituted	7	91	91	122	22.9**	ab/c
6	Ash of dried whole liver[6]	6	81	87	108	20.2**	ab/c
	Protein:						
7	Casein increased from 25 to 35%			94			
	Casein increased from 25 to 45%	6	98	89(b_1)	129	14.4**	abb$_1$/c
8	Methionine omitted from ration	10	91	93	117	23.2**	ab/c
9	Methionine increased 3×	8	101	90	124	16.4**	ab/c
10	Egg albumen substituted for casein	13	103	101	119	11.1**	ab/c
	Antibiotics:						
11	Aureomycin[7]	9	52[9]	56[9]	68[9]	4.6*	ab/c
12	Penicillin[7,8]	7	73	85	104	14.2**	ab/c

[1] See footnote 1, table 1.
[2] See footnote 3, table 2.
[3] Except choline, which when fed at 5× level caused a growth depression.
[4] Fed as fish liver oil.
[5] Jones and Foster ('42); modified to contain the following additional ingredients (μg/gm total salt mixture): $K_2Al_2(SO_4)_4 \cdot 24H_2O$, 92; NaF, 506.
[6] Equivalent to 15% dried whole liver.
[7] 100 mg/kg diet.
[8] Procaine penicillin "G" (1000 U./mg).
[9] Average 2-week gain in weight.

Workers at Wisconsin (Lewis et al., '50; Dietrich et al., '52) found that the substitution of dextrin for sucrose in a ration of the type used here stimulated growth of the hyperthyroid rat. In similar tests carried out in this laboratory (table 6, groups 1 and 2), dextrin was found to have no stimulatory effect.

Several workers[7,8] (Ershoff, '49; Greenburg and Deuel, '50; Westerfeld and Richert, '52; Stevens and Henderson, '58; Overby et al., '59b) found that, when hyperthyroid rats were fed a diet containing only a small amount of unsaturated fat, an increase in fat of this type in the ration overcame partially the depression in growth rate. Ershoff ('53) reported the addition of 10% of fat, along with B vitamins, to counteract completely the growth depression obtained with hyperthyroid rats. In some instances (Greenburg, '52; Overby et al., '59b) such action has been attributed to the effect of essential fatty acids. However, the data obtained by the latter workers for olive oil and butterfat seem to fit a curve based on total unsaturation (iodine number) better than they do a curve based on linoleic acid content *per se*.

Tests were carried out in this laboratory in which the level of cottonseed oil was increased beyond that included in the basal thyroprotein ration (10% fat). Substitution was made isodynamically; thus any positive response observed could not be attributable to a lowered thyroprotein intake. When the fat was increased by the approximate amount contained in 10% of dried whole liver (group 3, table 6), no response was obtained. When it was increased to 23 or 40% of the ration, however, increased growth was found in most instances, both with the basal ration alone and with the ration containing dried whole liver. The increase at the 40% level was no greater than that found at the 23% level. The response appeared to be separate and distinct from that obtained with liver, since the same response was obtained in the presence of liver as in its absence.

Substitution of butterfat or of margarine fat for cottonseed oil at the 10% level (group 6) failed to give decreased growth on the basal ration, which is contrary to what might be expected from its linoleic acid content or even unsaturated fatty acid content.

Part of the growth response obtained with liver residue has been attributed to its cholesterol content.[7] Conflicting results, however, have been obtained in regard to the effect of cholesterol and other steroids on the hyperthyroid rat[8] (Marx et al., '48; Ershoff and Marx, '48; Westerfeld and Richert, '52; Milcu et al., '56; Stevens and Henderson, '58; Overby et al., '59a).

In the present tests, summarized in groups 7 and 8, table 6, the inclusion of steroids in the ration gave significantly increased growth in only three of 7 experiments. This increase amounted on the average to about one third (zero to 50% in individual experiments) of that obtained by adding 10% of dried liver to the ration. No difference was observed between cholesterol and progesterone. Several levels of steroids were tested. None appeared to promote growth more than did 0.17% (the approximate level contributed to the diet by the liver). With the ration containing 10% of dried liver, no significant increases in growth resulted from the feeding of steroids. Thus it would appear, in agreement with the findings of Page et al.[7] that part of the response to liver can be accounted for by its steroid content, although the extent of the contribution seems to be quite variable from one experiment to another. The remainder of the liver activity must be accounted for by some other factor(s).

Combinations of cholesterol and increased fat were also tested (table 7). In this instance, adding these substances singly to the basal thyroprotein ration gave slight but not significant increases in weight gains. A combination of these two substances, however, gave pronounced increases in growth. With the ration containing 10% of dried whole liver (experiment 3), increasing the fat content by 13% led to improved growth which was not augmented by the addition

[7] Page, A. C., Jr., F. R. Koniuszy, D. E. Wolf, P. Aldrich and K. Folkers 1956 Factors in liver reversing thyroid stress in rats. Federation Proc., *15*: 568 (abstract).

[8] See footnote 6, p. 551.

TABLE 6

Activity of dextrin, increased fat and steroids for the hyperthyroid rat

Group	Test material	No. of litters	Average 3-week gain in weight					
			Basal ration	Basal ration + test material	Basal ration + 10% dried whole liver	Basal ration + 10% dried whole liver + test material	F[1]	Duncan's multiple range test[2]
			gm (a)	*gm* (b)	*gm* (c)	*gm* (d)		
	Dextrin:[3]							
1		13	83	84	—	—	—[4]	—
2		16	—	—	125	128	—[4]	—
	Fat increased to:[5]							
3	12%	5	66	69	93	—	13.6**	ab/c
4	23 or 40%	45	83	93	—	—	18.3**	a/b
5	23 or 40%	62	—	—	116	126	17.7**	c/d
	Fat changed from cottonseed oil to:							
6	Butterfat[6]			81		112		
	Margarine fat[7]	6	80	86(b_1)	117	109(d_1)	9.4**	abb$_1$/cdd$_1$
	Steroids:[8]							
7		57	91	100	—	—	17.7**	a/b
8		64	—	—	121	125	2.6	—

[1] See footnote 1, table 1.

[2] See footnote 3, table 2.

[3] Substituted for sucrose.

[4] Not significant; error mean square greater than treatment mean square.

[5] Cottonseed oil increased isodynamically from 10%. With 40% fat levels, in order to prevent fat separation, 3.5% ethyl cellulose was added or 1.5% soybean lecithin replaced 1.5% cottonseed oil.

[6] 10%; fed as whole butter.

[7] 10%; fed as oleomargarine.

[8] Either cholesterol, fed at levels from 0.17 to 2.0%, or progesterone, fed at 0.17 or 0.50%.

TABLE 7

Comparative activity of increased fat, steroids and dried whole liver

Group	No. of litters		Average 3-week gain in weight				F[1]	Duncan's multiple range test[2]
			No cholesterol		Cholesterol (0.5%)			
			10% fat	23% fat	10% fat	23% fat		
			gm	*gm*	*gm*	*gm*		
			(a)	(b)	(c)	(d)		
1	7	No liver	94[3]	98	—	122	10.1**	ab/d
2	7	No liver	76[3]	—	85	109	8.3**	ac/d
3	6	Liver[4]	128	142	—	146	3.9*	a/bd
4	6	No liver	89[3]	—	—	116	20.8**	$a/a_1d/d_1$
		Liver[4]	$122(a_1)$	—	—	$140(d_1)$		

[1] See footnote 1, table 1.
[2] See footnote 3, table 2.
[3] Basal thyroprotein ration.
[4] 10% Dried whole liver.

of cholesterol. In a direct litter-mate comparison (experiment 4), the increase brought about by the addition of liver was about 27% less when increased fat and cholesterol were present than when they were absent. Dextrin was also tested as a factor in this series of experiments but gave essentially the same results as sucrose. The results of the above experiments are in agreement with the previous conclusion that steroids can account for part of the activity of liver but that the action of the added fat is separate and distinct from that obtained with liver. Whether the action of the added fat in improving growth of the hyperthyroid rat is a direct one resulting from a need in the diet for unsaturated fat *per se* or an indirect one resulting from an influence of unsaturated fat on the synthesis of unidentified nutrients cannot be determined from these experiments.

In table 4 are shown tests of the effect of various crude food materials upon growth of the hyperthyroid rat when these materials were added to the basal ration. Many of these same substances were also tested by incorporating them in the basal ration along with dried whole liver (groups 1 through 8, table 8). The level of liver fed (10%) gave the maximum growth obtainable with this supplement in the tests shown in groups 6 and 7, table 3, and indeed with all the other lots tested at different levels, with but one exception. Many of the groups in table 8 comprise a number of experiments carried out at widely separated periods of time and with a number of different lots of dried liver. Thus, even though variations may occur in the activity of different lots of liver, it would appear that the growth obtained with the liver control groups at the 10% level represent the maximum or nearly maximum growth obtainable with this supplement.

With the liver-supplemented ration, known nutrients tested were without effect. Increases in vitamin or protein content (groups 9–12) or the substitution of egg albumen for casein (group 13) failed to give additional growth. Antibiotics (Aureomycin, penicillin), lyxoflavin, orotic acid, purines (xanthine, guanine,

TABLE 8

Effect on growth of adding various substances to the basal thyroprotein ration containing 10% dried whole liver

Group	Number of experiments	Test material	No. of litters	Average 3-week gain in weight		t[1]
				Basal ration + 10% dried whole liver	Basal ration + 10% dried whole liver + test material	
				gm	*gm*	
1	3	Corn meal, yellow (10%)	24	112	117	1.3
2	7	Cottonseed meal (10%)	77	105	117	6.1**
3	3	Fish meal (10%)	24	122	137	4.7**
4	1	Fish solubles (4%)	10	117	119	0.2
5	2	Soybean oil meal (10%)	16	105	116	3.3**
6	4	Soy protein, brand 1 (10%)	37	96	110	3.8**
7	1	Soy protein, brand 2 (10%)	10	107	110	0.3
8	1	Yeast, dried brewers' (10%)	8	110	106	0.6
		Vitamins:				
9	1	All in mixture doubled	8	123	123	0.0
10	3	All increased 5× (except choline)	26	106	103	0.7
11	1	Vitamins A and D doubled[2]	9	105	103	0.4
		Protein:				
12	1	Casein increased to 35%	8	125	127	0.4
13	2	Egg albumen substituted for casein	15	118	111	1.2

[1] See footnote 1, table 1.
[2] Fed as fish liver oil.

adenine) and thioctic acid were also inactive.

On the other hand, cottonseed meal, fish meal, soybean oil meal, or one brand of soy protein, when fed at 10% of the ration, gave significantly increased growth above that obtained with 10% of dried whole liver alone, whereas corn meal, fish solubles, another brand of soy protein or yeast did not. Other substances tested and found to be without activity under these conditions included various milk products, such as dried skim milk, cheddar cheese, dried cheese whey and butter (substituted for an equal amount of cottonseed oil). A study of the individual experiments summarized in groups 1 through 8 indicated that the same conclusions as to relative activity of the various supplements would be drawn in 20 of these 22 experiments as were drawn from a study of the data as presented in the table, the only exceptions being one experiment in the cottonseed meal group and one in the soy protein group. If the results of the tests in table 8 are compared with those of table 4, certain differences will be observed. Thus soybean oil meal was found to be completely inactive in the absence of liver but to promote increased growth in its presence; soy protein (brand 1) at the 10% level also showed greater activity in the presence of liver. On the other hand, dried brewers' yeast, which in the absence of liver gave approximately one-half the growth obtained with liver, was completely inactive when fed along with this substance. The above results suggest that two different unidentified nutrients are involved and that, accordingly, the hyperthyroid rats used in these tests require at least two still unidentified nutrients, some food substances such as yeast containing one or the other and other food materials such as cottonseed meal containing both.

TABLE 9

Effect of various supplements on the attainment of normal growth by the hyperthyroid rat

Group	Test material	No. of litters	Average 3-week gain in weight				F[1]	Duncan's multiple range test[2]
			Basal ration	Basal ration + 10% dried whole liver	Basal ration + 10% dried whole liver + test material	Basal ration without iodinated casein		
			gm (a)	gm (b)	gm (c)	gm (d)		
1	Mixture 1[3] Mixture 2[4]	20	74[5]	106	125 125(c_1)	138	16.7**	b/cc$_1$/d
2	Cottonseed meal (10%)	10	79[6]	118	133	149	20.4**	b/c/d
3	Fat increased to 23%[7] Cottonseed meal (10%) Cottonseed meal (10%); fat increased to 23%[7]	8	—	124	138 139(c_1) 153(c_2)	—	14.1**	b/cc$_1$/c$_2$
4	Fat increased to 23%[7] Fish meal (10%) Fish meal (10%); fat increased to 23%[7]	8	—	135	140 148 146	—	2.5	—
5	(Holtzman rats)	5	110	161	—	174	21.4**	a/bd

[1] See footnote 1, table 1.

[2] See footnote 3, table 2.

[3] Ration altered by replacing sucrose with dextrin, replacing casein by soy protein (same protein level), increasing methionine to 0.4%, adding 6% fish meal, 4% cottonseed meal, 0.5% cholesterol.

[4] Ration altered by replacing sucrose with dextrin, replacing casein by soy protein (same protein level), increasing methionine to 0.4%, reducing dried whole liver to 4%, addition of 1% sulfathalidine, 4% fish meal, 4% soybean meal and 4% cottonseed meal.

[5] Average of rats from 13 litters; not included in statistical calculation.

[6] Average of 4 surviving rats; not included in statistical calculation.

[7] Isodynamically.

Even when one or more of these active substances was added to the basal ration along with 10% of dried whole liver, growth equal to that found by omitting thyroprotein from the ration was still not obtained (groups 1 and 2, table 9). In group 1, the ration was modified by the incorporation of a number of active materials, whereas in group 2, cottonseed meal alone was used. In both cases, the growth resulting was better than that found with 10% of liver but not as good as that using the basal ration without thyroprotein.

As discussed previously, increased growth could be obtained on the liver ration by increasing the fat level to 23%. None of the above active materials—cottonseed meal, fish meal, and others—when fed at 10%, increased the fat level of the ration by as much as 2% which, judging from the test in group 3, table 6, would not change growth appreciably. The effect of raising to 23% the fat level of a ration containing 10% of dried whole liver and 10% of cottonseed meal or fish meal are shown in groups 3 and 4. The results in group 3 indicate that the effects of cottonseed meal and additional fat are separate and additive. In experiment 4, the results are less clear since the weight gains are not statistically different. Fed separately, fish meal tended to give somewhat greater growth than increased fat, whereas the two together gave no better growth than fish meal alone. Although no direct comparison was made in these experiments, the growth obtained using a combination of dried liver, cottonseed meal and higher fat or of liver and fish meal was about as good as could be expected from the ration with iodinated casein omitted.

One test shown in group 5, table 9, was made using the Holtzman strain of rats. Similar although not identical results were obtained with this strain and the Beltsville strain. From a comparison of group 5 with groups 1 and 2, it is apparent that with Holtzman rats, 10% of dried whole liver overcame the depression with thyroprotein to a greater extent than it did with Beltsville rats.

SUMMARY

When rats fed a purified ration containing all known nutrients were rendered hyperthyroid, their growth rate was considerably decreased because of a deficiency in the ration of certain unidentified nutrients. Evidence was presented suggesting that crude food materials contain at least two different unidentified nutrients, some food substances containing one or the other of these nutrients and other food materials containing both. Part but not all of the activity of dried whole liver could be accounted for by its steroid content. Unsaturated fat alleviated partially the growth depression. Its activity appeared to be distinct from that of dried whole liver and probably separate from both of the unidentified nutrients mentioned above.

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